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**14. ABSTRACT** The development of breast cancer is associated with gene amplification and overexpression that are believed to have a causative role in oncogenesis. An important challenge in breast cancer research is to identify and characterize these genetic changes. Focal amplifications involving chromosome 8p11-p12 occur in approximately 15~20% of primary, uncultured human breast cancers. Recently, we have undertaken a detailed genomic and expression analysis of the 8p11-p12 amplicon in breast cancer cell lines and identified several novel candidate genes including TC-1 and FLJ14299. We observed that TC-1 is located at the common core-amplified domain of the 8p11-12 region and overexpressed in the subset of breast cancer cells. Furthermore, we have found that TC-1 has properties of an oncogene: TC-1 expression in normal mammary epithelial cell line MCF10A increases growth rate and allows growth in soft agar. Notably, suppression of TC-1 expression by siRNA inhibited cell proliferation in TC-1 over expressing breast cancer cell lines. Our recent date also suggested that TC-1 over-expression is associated with the enhanced expression of a subset of beta-catenin target genes in breast cancer.

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Zeng-Quan Yang, Ph.D.
Final Report for Award DAMD 17-03-1-0459
May 31, 2007

**Introduction:**

The development of breast cancer is associated with gene amplification and overexpression that are believed to have a causative role in oncogenesis(1-4). An important challenge in breast cancer research is to identify and characterize these genetic alternations. Global genomic and transcriptomic analysis have led to important insights in our understanding of the complexity and heterogeneity of this disease, and providing new avenues for the discovery of genetic alternation in human breast cancer (HBC). Recently we have performed the cytogenetic and molecular genetic profile of the SUM panel breast cancer cell lines. Of specific relevance to this application, we have found that three cell lines have overlapping amplicons in the short arm of chromosome 8. From this amplicon, we identified several novel candidate genes including TC-1 (C8ORF4), FLJ14299 and others(5). TC-1 is a novel gene highly expressed in thyroid cancer and some fraction of breast cancers. FLJ14299 contains a C2H2-like motif, which is also present in several tumor-related genes. Aberrant expression of TC-1 and FLJ14299 could be related to development and progression in breast cancer. The objective of this proposal is to characterize two novel genes *TC-1* and *FLJ14299* of the 8p11-12 amplicon in HBC, and to examine the prognostic and predictive significance of 8p11-12 amplification and overexpression pattern in primary breast cancer. The hypothesis is that the 8p11-12 amplicon contains multiple overexpressed genes which could act, either alone or in combination, to influence breast cancer development. The specific aims as outlined in this proposal will help us to better understand the biological function and genetic pathway of new target genes of 8p11-12 amplicon and identify better prognostic and predictive markers for an important subset of breast cancer.

**Body:**

*Task 1*. To test the mechanistic significance of two novel genes, *FLJ14299* and *TC-1,* found to be amplified and over expressed in the SUM-44, SUM-52 and SUM-225 breast cancer cell lines (Months 1-20)

In our previous annual report, we demonstrated that TC-1 has oncogenic properties. Overexpression of TC-1 in a spontaneously immortalized normal mammary epithelial cell line, MCF10A, enhanced cell proliferation in tissue culture. More important, TC-1 overexpression significantly promoted colony formation in soft agar assay relative to control vector alone (Figure 1). These results support the notion that TC-1 can facilitate cell growth and anchorage-independent growth associated with transformed phenotypes. Overexpression of FLJ14299 in MCF10A cells did not enhance cell proliferation and anchorage-independent growth. In addition, suppression of TC-1 expression by siRNA inhibited cell proliferation in TC-1 over expressing breast cancer cell lines.

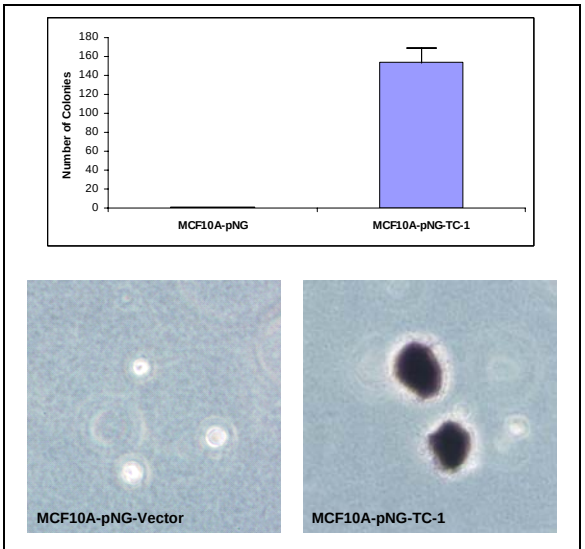


Figure 1: Number and representative pictures of soft-agar colonies in pNG empty vector infected cells and cells stably expressing TC-1. MCF10A-pNG-TC-1 and pNG-Vector Cells were grown for 3-4 weeks in soft agar and stained with the vital dye 2-iodonitrotetrazolium violet.

In order to investigate the individual and cooperating oncogenic properties of newly identified genes in 8p11-12 amplicon, we established a cDNA expression library containing 8 candidate genes including TC-1 and FLJ14299 using a lentiviral expression system in this year. We transduced cells with several different combinations of genes from the lentiviral library into MCF10A cells and selected recipient cells in serum-free medium lacking EGF or IGF. RT-PCR with genes and vector-specific primers were used to detect

transduced genes in cell clones that acquired EGF independence. Our data revealed that a combination of TC-1 and one of other two genes was sufficient to induce EGF independent growth. Clones containing only one of the three aforementioned genes were unable to survive in EGF-free medium. This result suggested that cooperative activity of oncogenes including TC-1 within amplicons is crucial for inducing the specific transforming phenotypes in breast cancer.

*Task 2*. To detect potential downstream target genes of *TC-1*and *FLJ14299* overexpression using human cancer array and to investigate their particular tumor-related biological phenomena (Months 8-48)

*TC-1* is a novel gene that was originally cloned from suppression subtractive hybridization between papillary thyroid carcinoma and its surrounding normal thyroid tissue(6). Recently, the structural characterization of the TC-1 protein revealed it is a natively disordered protein(7). Disordered proteins have been suggested to play roles in cell-cycle control, signal transduction, transcriptional and translational regulation. Recently, Jung et al. have reported that TC-1 is a positive regulator of the beta-catenin pathway in gastric cancer. TC-1 interacts with Chibby (Cby), which negatively regulates beta-catenin–mediated transcription. Thus, TC-1 enhances beta-catenin signaling by relieving the suppression induced by Cby.

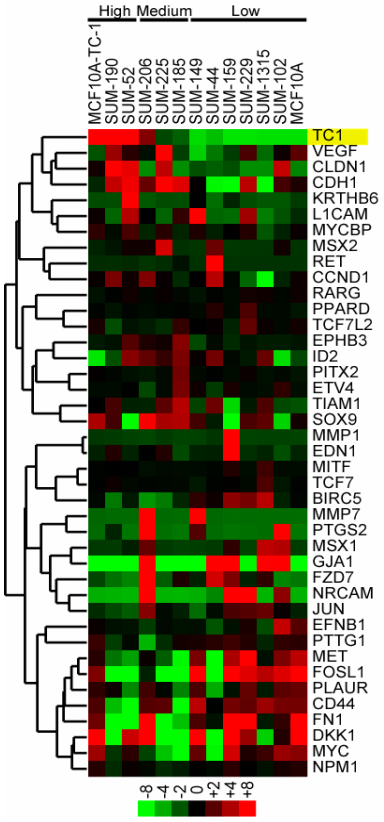


Figure 2: Unsupervised hierarchical clustering of *TC-1* and 41 Wnt/β-catenin target genes in our 11 SUM-breast cancer cell lines, MCF10A cells and MCF10A-TC-1 cells. Each row represents a target gene, and each column represents a cell line. Color codes are indicated at the bottom of the figure.

Since TC-1 has been implicated as a modulator of Wnt/ β-catenin signaling, we tested whether TC-1 over expression is associated with an increased expression of β-catenin target genes in our 11 SUM-breast cancer cell lines, MCF10A-pNG-TC-1 cells and MCF10A cells. Gene expression profiles were obtained using Affymetrix U133A arrays, which include 122 probe sets for 58 Wnt/ β-catenin pathway target genes. These target genes were identified from a review of the literature, and from those listed on the Nusse laboratory website (http://www.stanford.edu/~rnusse/wntwindow.html). Probe sets measuring expression for a given transcript were averaged and then used as parameters to generate a generalized linear model with *TC-1* expression as a logistic response. This test can find significant associations between TC-1 expression and many subtle gene expression changes. When considered together in this way, the gene expression patterns of these 58 genes did not significantly associate with *TC-1* gene expression levels ($p < 0.075$).

The 41 Wnt/ β-catenin target genes whose average expression was greater than 8 intensity units were subjected to hierarchical clustering (Figure 2). Clearly, no discernable overall pattern of expression relative to *TC-1* expression was evident. Nevertheless, several individual β-catenin target genes were found to be highly expressed in all but one breast cancer cell line that exhibited TC-1 over expression including *VEGF*, *CLDN1* and *CDH1*. Up-regulation of *CLDN1* and *CCND1* in TC-1 over expressing cells was verified by Q-RT-PCR analysis. In addition, there was a sub-cluster of genes whose expression was inversely correlated with TC-1 expression. Interestingly, both MCF10A-TC-1 cells and SUM-206 cells have high and medium levels of TC-1 expression, but do not cluster with the other TC-1 over expressing cells. The mechanisms controlling expression of β-catenin target genes are complex and involve other signaling pathways and transcription regulatory factors. Given that there was no association of TC-1 over expression with global expression of β-catenin target genes, and the lack of direct evidence for β -catenin signaling in SUM-52 cells, the high expression of some β -

catenin target genes observed in our experiments likely occurs independently of β -catenin signaling, but in a manner that is regulated by TC-1.

*Task 3*. To determine the amplification and overexpression pattern of 8p11-12 genes in primary breast cancer and to determine their associations with tumor phenotype and prognosis (Months 6-48)

To determine the amplification pattern of 8p11-12 genes in primary breast cancer, quantitative PCR analysis was carried out using genomic DNA obtained from 90 breast cancer specimens. The PCR experiments were performed using primers specific for *FLJ14299*, *LSM1*, *FGFR1* and *TC-1*, as these genes span the 8p11-12 amplicon detected in the cell lines(5). Of the 90 breast cancers examined, 25 showed evidence of high level amplification (greater than 4-fold) in at least part of the 8p11-12 region. Interestingly, *TC-1* and *FLJ14299* were most commonly amplified, while *FGFR1* was only found to be greater than 4-fold amplified in 4 of 90 primary breast cancers. These results suggest that genes such as TC-1 and FLJ14299 flanking the *FGFR1* locus may be of greater significance in breast cancers.

To elucidate the significance of TC-1 expression in primary HBC, we performed quantitative RT-PCR experiments using RNA isolated from the primary HBC specimens. In 29 samples analyzed, 7 samples had TC-1 gene amplification by array CGH, and/or quantitative genomic PCR. In these 29 samples,thirteen samples (45%) exhibited an up-regulated of TC-1 mRNA level (>4 fold) when compared with the normal breast tissue (Figure 3). We did not find that the mRNA level correlated positively with the DNA amplification level in both breast cancer cell lines and primary HBC specimens. There were no clear clinical features that were associated with TC-1 mRNA expression level.

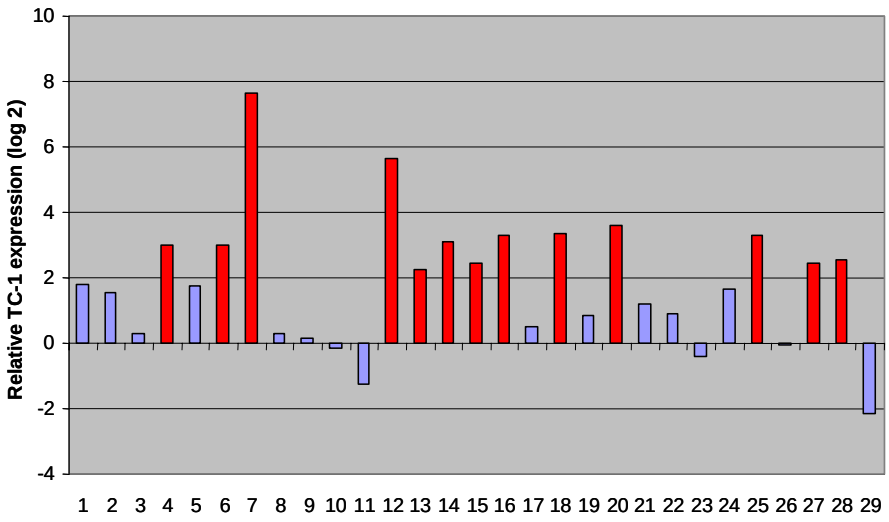


Figure 3: The graphs show the TC-1 mRNA expression level 29 primary breast cancer specimens. Gene expression levels (log2) relative to normal human breast tissue were obtained from quantitative RT-PCR after normalization to a housekeeping gene (GUSB).

**Training accomplishments:**

With the help of this training award, my ability in critical thinking and problem solving in research work, the skills needed to formulate and carry out research, and to report findings in peer-reviewed journals have been dramatically improved. From October 1, 2004, I became an Research Assistant Professor in the Department of Pathology, Wayne State University School of medicine, and a member of the Breast cancer research program of the Karmanos Cancer Center.

**Key Research Accomplishments:**

1, Novel gene TC-1 of 8p11-12 amplicon has oncogenic properties in breast cancer.

2, TC-1 induced a subset of beta-catenin target genes including CCND1, CLDN1, EDN1, MITF and MMP7 in breast cancer.

3, TC-1 mRNA levels were frequently up-regulated in breast tumors.

**Reportable Outcomes:**

*Manuscripts:*

1. **Yang Z-Q**, Moffa A, Haddad R and Ethier S. Transforming properties of TC-1 in human breast cancer: Interaction with FGFR2 and β-catenin signaling pathways. International Journal of Cancer. Epub ahead of print. 2007
2. **Yang, Z-Q,** Streicher KL, Ray, M, Abrams J and Ethier, S. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. Cancer Research, 66:11632-43. 2006
3. **Yang Z-Q**, Albertson D and Ethier S. Genomic organization of the 8p11-12 amplicon in three breast cancer cell lines. Cancer Genetics and Cytogenetic. 155:57-62, 2004

*Abstracts:*

1. **Yang, Z-Q.** Identification and characterization of novel oncogenes in breast cancer. Medicine in the Genomics Era, Post Genomics Summit 2006. Beijing, China May 17-19, 2006 (Oral presentation)
2. Ethier, S**,** Streicher KL, Ray, M, Abrams J and **Yang, Z-Q.** Cooperation of multiple oncogenes from the chromosome 8p11-12 amplicon in breast cancer. 2006 American Association for Cancer Research Annual Meeting in Washington, DC, April 1-5, 2006
3. **Yang Z-Q**, Dombkowski A, Streicher K,Ray M and Ethier S. Integrative genomic analysis identify novel oncogenes in breast cancer. 2006 American Association for Cancer Research Annual Meeting in Washington, DC, April 1-5, 2006
4. **Yang Z-Q**, Ray M, Grewal N and Ethier S. Identification and characterization of TC-1: a novel oncogene in human breast cancer, 2005 American Association for Cancer Research Annual Meeting in Anaheim, CA. April 16-20, 2005
5. Streicher K, **Yang Z-Q** and Ethier S. Over-expression of Lsm1/Casm in human mammary epithelial cells leads to growth factor independence. 2005 American Association for Cancer Research Annual Meeting in Anaheim, CA, April 16-20, 2005
6. **Yang Z-Q**, Ray M, Grewal N and Ethier S. Characterization of the 8p11-12 amplicon in breast cancer, Era of Hope Department of Defense Breast Cancer Research Program Meeting in Philadelphia, Pennsylvania, June 8-11, 2005
7. **Yang Z-Q**, Ray M, Albertson D and Ethier S. Characterization of the novel candidate oncogene TC-1 in breast cancer and knockdown using siRNA. 2004 American Association for Cancer Research Annual Meeting in Orlando, Florida. March 27-31, 2004.
8. **Yang Z-Q**, Ray M, Albertson D, Kleer C and Ethier S. Genomic organization of the 8p11-12 amplicon in human breast cancer. SKCC Genomics, Signaling and Tumor Targeting Conference in San Diego CA, February 16-18, 2004( Oral and poster presentation)

**Conclusions:**

In summary, our study revealed that TC-1 is a transforming gene when over expressed in human breast cancer cells. In some cases, TC-1 is downstream of FGFR2 mediated signaling pathways. TC-1 over expression is also associated with expression of some β-catenin target genes, which may play an role in mediating the expression of transformed phenotypes. As a novel regulator of these pathways, TC-1 may be implicated in regulating the biological behavior of breast cancer through coordinated activation of key signaling molecules known to play a role in cell.

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**APPENDICES**

1. **Yang Z-Q**, Moffa A, Haddad R and Ethier S. Transforming properties of TC-1 in human breast cancer: Interaction with FGFR2 and β-catenin signaling pathways. International Journal of Cancer. Epub ahead of print. 2007
2. **Yang, Z-Q,** Streicher KL, Ray, M, Abrams J and Ethier, S. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. Cancer Research, 66:11632-43. 2006
3. **Yang Z-Q**, Albertson D and Ethier S. Genomic organization of the 8p11-12 amplicon in three breast cancer cell lines. Cancer Genetics and Cytogenetic. 155:57-62, 2004

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# Transforming properties of TC-1 in human breast cancer: Interaction with FGFR2 and β-catenin signaling pathways

**Zeng-Quan Yang*, Allison B. Moffa, Ramsi Haddad, Katie L. Streicher and Stephen P. Ethier***

*Breast Cancer Program, Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI*

**Breast cancer development is associated with gene amplification and over expression that is believed to have a causative role in oncogenesis. Previous studies have demonstrated that over expression of *TC-1*(*C8orf4*) mRNA occurs in ~50% of breast cancer cell lines and primary tumor specimens. Here, we show that TC-1 has transforming properties in human mammary epithelial (HME) cells and its expression is mechanistically linked to FGFR signaling cascades. *In vitro* experiments demonstrate that TC-1 over expression mediates both anchorage-independent and growth factor-independent proliferation of HME cells. *TC-1* was down regulated by the FGFR inhibitor PD173074 in the breast cancer cell line SUM-52 that also has an FGFR2 gene amplification and over expression. Furthermore, forced expression of FGFR2 in HME cells increased the level of expression of endogenous *TC-1* mRNA. TC-1 has been implicated as a modulator of Wnt/β-catenin signaling in 293 cells and in gastric cancer cells. However, while we did find increased expression of a subset of β-catenin target genes in TC-1 over expressing cells, we did not find an association of TC-1 with global expression of β-catenin target genes in our cells. Taken together, our data suggest that TC-1 over expression is transforming and may link with the FGFR pathway in a subset of breast cancer.**


**Key words:** TC-1; FGFR2; breast cancer

The development of breast cancer is associated with gene amplification and over expression, which is believed to have a causative role in oncogenesis.[1–5] *TC-1*(*C8orf4*) was one of the over expressed genes in our previous detailed genomic analysis of the 8p11-12 amplicon in human breast cancer (HBC) cell lines and tumors.[6,7] *TC-1* was originally cloned from the subtractive hybridization between a papillary thyroid carcinoma and its surrounding normal thyroid tissue. Subsequently, over expression of *TC-1* in thyroid cancer was found in 15 of 16 paired primary samples.[8] In addition, TC-1 expression had strong correlations with aggressive biological behavior and poor clinical outcome in gastric cancers.[9] The structural characterization of the TC-1 protein revealed it to be a natively disordered protein.[10] Disordered proteins have been shown to play roles in cell-cycle control, signal transduction, transcriptional and translational regulation and in macromolecular complexes such as ribosomes.[11–13]

The FGFR family has been found to play an important role in numerous types of human cancer, including breast cancer.[14] Amplification and over expression of *FGFR2* has previously been reported in 5% of primary breast cancer specimens.[14,15] SUM-52 is a breast cancer cell line isolated in our laboratory that grows under serum-free and epidermal growth factor(EGF)-free conditions, and has FGFR2 amplified and highly over expressed at both the mRNA and protein level.[16–18] Our previous studies suggest that amplification and over expression of FGFR2 can drive cellular phenotypes expressed in the SUM-52 breast cancer cell line, and in other breast cancer cells. Small molecule inhibitors that block signaling from this receptor can specifically reverse the transformed phenotype. In addition, alternatively spliced FGFR2 variants that differ in their transforming properties were isolated from SUM-52 cells.[16–18] Our early studies also demonstrated that *TC-1* was expressed at high levels in the SUM-52 breast cancer cell line.[7] Interestingly, in the present study we found that *TC-1* over expression is regulated by FGFR2 signaling in SUM-52 cells. These findings prompted further investigation of the transforming properties of TC-1 in breast cancer cells.

Recently, Jung *et al*. reported that TC-1 is a positive regulator of the β-catenin pathway in gastric cancer. TC-1 interacts with Chibby (Cby), which negatively regulates β-catenin mediated transcription.[19–21] Thus, TC-1 enhances β-catenin signaling by relieving the suppression induced by Cby. The expression of TC-1 was also analyzed using tissue microarray in correlation with β-catenin target genes in 299 gastric cancers. These experiments suggested that TC-1 correlates strongly with the expression of a subset of β-catenin target genes, including CCND1, LAMC2, MMP7 and MMP14.[9] However, the relevance of TC-1 over expression in the development and progression of breast cancer has not yet been elucidated. In our present work, we found that TC-1 has transforming properties in human mammary epithelial (HME) cells and its expression can be mechanistically linked to the FGFR2 pathway in some breast cancers. While the expression of some β-catenin target genes was influenced by the level of TC-1 expression, most β-catenin target genes were not changed in TC-1 over expressing breast cancer cells, or in TC-1-transformed HME cells. Thus, TC-1 has transforming properties in HBC cells, which are independent of effects on β-catenin signaling, but which are linked to FGFR2 signaling when the gene is amplified and over expressed.

## Material and methods

### *Cell lines and tumor specimens*

The culture of the SUM series of HBC cell lines and normal mammary epithelial cell line MCF10A have been described in detail previously.[22] Primary HBC specimens were obtained from Asterand (Detroit, MI).

### *Northern analysis and quantitative RT-PCR reactions*

Total RNA was prepared from cultured HBC cell lines and the HME cell line (MCF10A) by standard methods. Northern blot hybridization was performed as described previously.[23] For quantitative RT-PCR reactions, RNA was converted into cDNA *via* a reverse transcription reaction using random hexamer primers. Primers and probes were ordered from Applied Biosystems (Foster City, CA) Assays-by-Design service. GAPDH primer set was used as a control. Quantitative RT-PCR was done in 25-μL reactions, in

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96-well plates, using the Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Interpretation of relative expression data was calculated as described by Livak and Schmittgen.[24]

*Retrovirus and lentivirus construction and transduction of cells*

pNG-TC-1 retrovirus construction was done by ligating the full-length *TC-1* into the *Xho*I-*Eco*RI-digested pNG3000 retroviral vector. The construct was sequenced to ensure that the sequences and orientation were correct. MCF10A and NIH3T3 cells were infected with virus in the presence of polybrene for 24 hr. The polyclonal populations of cells resistant to Puromycin were selected and tested for the expression of the insert genes either by Northern blot and/or Q-RT-PCR.

To generate lentiviral expression constructs containing the *TC-1* gene (pLenti-TC-1), we first created an entry clone containing the full-length TC-1 using pENTR directional TOPO cloning kit (Invitrogen, Carlsbad, CA). After we have generated the entry clone, we performed the LR recombination reaction to transfer the gene into the pLenti-DEST vector to create the expression clone. The construct was sequenced to ensure that the sequences and orientation are correct. Producing lentivirus was performed by cotransfecting the 293FT cell line with the pLenti expression construct and the optimized packaging mix (Invitrogen, Carlsbad, CA). The HME cell line MCF10A was transduced with lentivirus. Control infections with pLenti-LacZ virus were performed in parallel with plenti-TC-1 infections. Selection began 48 hr after infection in growth medium with 10 μg/mL blasticidin in the absence of either insulin or EGF. Upon confluence, selected cells were passaged and serially cultured.

*TC-1 siRNA*

Two siRNA sequences corresponding to human TC-1 sequences starting at the 100th base (denoted TC-1-siRNA100) and 175th base (denoted TC-1-siRNA175) in the coding sequence were synthesized. A nonspecific, control oligonucleotide was also obtained from QIAGEN (Cat. 1027280, QIAGEN, Valencia, CA). Transfection into the SUM-52 and SUM-190 breast cancer cell lines was carried out using TransMessenger Transfection Reagent and/or RNAiFect Transfection reagent (QIAGEN, Valencia, CA) with double-stranded RNA at a final concentration of 100 nmol/L. The samples for Q-RT-PCR and Northern blot analysis were taken at the indicated time points.

*FGFR inhibitor experiments*

For the FGFR inhibitor experiments, SUM-52 cells were grown in their normal growth medium supplemented with the FGFR inhibitor PD173074 (dissolved in DMSO) at a final concentration of 1 μM. PD173074 was provided by Dr. Wilbur Leopold and Pfizer Pharmaceuticals (Ann Arbor, MI). Growth media for control cells were supplemented with equivalent volumes of DMSO with no inhibitor.

*Soft agar assays, morphogenesis assay and proliferation assays*

Soft agar assays were performed as previously described.[16] Briefly, six-well dishes were coated with a 1:1 mix of the appropriate 2× medium for the cell line being studied and 1% Bactoagar. Cells were plated at $1 \times 10^5$ cells per well in a mixture of appropriate medium and 0.3% Bactoagar. Cells were fed 3 times per week for 3–4 weeks, stained with 500 μg/mL *p*-iodonitrotetrazolium violet (Sigma, St Louis, MO) overnight, photographed and counted on an Accucount 1000 (Fisher Scientific, Pittsburgh, PA). Soft agar assays were done in duplicate and repeated at least twice. The three-dimensional morphogenesis assays in matrigel was performed as described.[25]

To determine cell proliferation, cells were seeded in 6-well plates at $3 \times 10^4$ cells per well on Day 0. Cells were counted in triplicate on Day 1 and every 3 days thereafter using a Coulter Counter (Miami, FL).

*cDNA microarray and bioinformatics analysis*

RNA was isolated using a Qiagen RNeasy kit. DNA microarray analysis of gene expression using a 20,000 element cDNA array in SUM-52 cells after exposure to PD173074 for 24 hr was carried out as described previously.[26] For Affymetrix array, a linear amplification protocol, consisting of 1 round of double-strand cDNA synthesis and *in vitro* transcription, was used. The labeled cRNA (10 μg total) was fragmented and used in the hybridization reaction to GeneChip probe array HG U133A according to the manufacturer's protocol. Each sample was run and the resulting cel files created by Affymetrix MAS5 were analyzed with the open source R Statistical Package (www.r-project.org) using libraries from the Bioconductor Project (www.bioconductor.org).[27] Quantile normalized gene expression levels were summarized using a sequence specific expression model provided by the Bioconductor library 'gcrma'.[28] Discriminant analysis of TC-1 over expressing cells relative to TC-1 nonexpressing cells was performed using a moderated analysis of variance linear model of gene expression implemented in the Bioconductor library 'limma'.[29] TC-1 over expressing cell lines included SUM52, SUM190 and MCF10A-TC-1. TC-1 nonexpressing cell lines included SUM44, SUM102, SUM149, SUM159, SUM229, SUM1315 and MCF10A.

Probability values were corrected with the use of the false discovery rate method. The association of TC-1 over expression with Wnt/β-catenin-responsive gene expression was determined used the Bioconductor package 'globalTest'.[30] First, the 122 probe sets for 58 Wnt/β-catenin pathway target genes were first averaged across probe sets measuring the same transcript. These gene expression measurements were used as parameters in a generalized linear model where TC-1 expression was a logistic response. The regression coefficients were then analyzed using a goodness of fit test to determine association, as previously described.[30]

## Results

*TC-1 is expressed at high levels in breast cancer cell lines and in primary human tumor specimens*

Previously, we used a combination of array comparative genomic hybridization, fluorescence *in situ* hybridization and Southern blot hybridization to perform a detailed genomic analysis of an 8p11-12 amplicon in 3 breast cancer cell lines: SUM-44, SUM-52 and SUM-225.[6,7] *TC-1*, which is located within the common core-amplified domain of the 8p11-12 region, was significantly amplified in these 3 cell lines. To assess the level of *TC-1* expression in breast cancer, we performed mRNA expression analysis of *TC-1* in our panel of 11 breast cancer cell lines and the immortalized, nontransformed mammary epithelial cell line (MCF10A cells). *TC-1* expression levels were measured using both Affymetrix GeneChip microarrays and Northern analysis and were subsequently compared relative to the immortalized HME cell line MCF10A. The results of these experiments demonstrate that *TC-1* is very highly over expressed (>100-fold increase) in 2 breast cancer cell lines, SUM-52 and SUM-190, and is moderately over expressed (4–100-fold change) in 4 other lines (Table I). These results were verified by northern blot hybridization (Fig. 1) and quantitative real time RT-PCR (Q-RT-PCR). In addition, *TC-1* is over expressed in ∼50% of primary breast cancer specimens as reported recently by our group.[31] Of the 29 tissue samples analyzed by Q-RT-PCR, thirteen specimens (45%) exhibited TC-1 over expression at the mRNA level (>4-fold increases) when compared to normal breast tissue.[31] Thus, the TC-1 gene was over expressed in multiple cell lines and in nearly half of our panel of breast cancer samples.

*TC-1 has transforming properties* in vitro

Given that *TC-1* was amplified and over expressed in multiple cell lines and in approximately half of the breast cancer tissue samples, we addressed the question of whether TC-1 possesses



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AQ1
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**TABLE I** – TC-1 EXPRESSION LEVELS FOR 11 SUM BREAST CANCER CELL LINES USING OUR AFFYMETRIX ARRAY DATABASE

| Cell line | High-level overexpression | | Middle-level overexpression | | | No overexpression | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | SUM-190 | SUM-52 | SUM-206 | SUM-225 | SUM-185 | SUM-44 | SUM-149 | SUM-1315 | SUM-229 | SUM-159 | SUM-102 |
| Fold change | 168.7 | 121.7 | 31.5 | 11.8 | 7.4 | −1 | 1.8 | −1.4 | −1.3 | −1.2 | −2.1 |

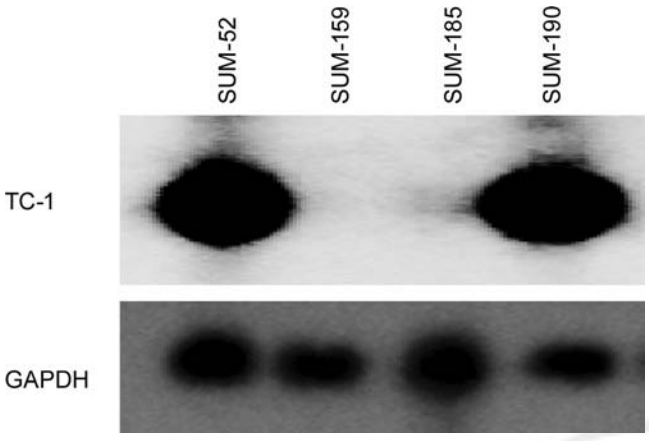


**FIGURE 1** – Representative Northern blot analysis demonstrates high-level *TC-1* over expression in SUM-52 and SUM-190 cell lines. *GAPDH* was used as the loading control probe in Northern blot hybridization.

transforming properties. To this end, we established 1 mouse cell line (NIH3T3-pNG-TC1) and 2 human cell lines (MCF10A-pNG-TC-1 and MCF10A-pLenti-TC-1), all of which stably over express *TC-1* under the control of the CMV promoter. To avoid clonal selection effects, all experiments were performed with uncloned and drug-selected pools of cells that stably over express TC-1. The mRNA expression level of *TC-1* in these 3 uncloned cell lines was measured with Q-RT-PCR (Fig. 2*a*). TC-1 over expressing cells and their respective parental cell lines were assayed for alterations in growth rates, for growth factor-independent proliferation and for anchorage-independent growth.

F2

First, the ability of TC-1 to enhance the growth of cells was investigated. NIH3T3-pNG-TC-1 cells had an accelerated growth rate relative to the parental cells or cells transfected with the empty vector. Seven days after equal numbers of cells were plated, there were ~5 times as many NIH3T3-pNG-TC-1 cells as NIH3T3-pNG empty vector cells (Fig. 2*b*).

Next, growth factor-independent proliferation of TC-1-over expressing cells was assayed. We tested MCF10A-TC-1 cells for their ability to grow in the absence of either insulin-like growth factor, EGF or the absence of both growth factors. While vector control MCF10A cells require EGF and insulin-like growth factors for continuous growth, cells transduced with pNG-TC-1 and pLenti-TC-1 grew continuously in the absence of insulin-like growth factors for many passages and are now routinely cultured in this medium (Fig. 2*c*). However, over expression of TC-1 did not induce EGF independence in MCF10A cells.

Finally, MCF10A-pNG-TC-1 and pLenti-TC-1 cells were assayed for anchorage-independent growth. Both cell lines grew as colonies in soft agar, a property not observed in the parental MCF10A cells or in MCF10A cells containing the empty vector (Fig. 2*d*). To further examine the effects of TC-1 activity in a context that more closely resembles *in vivo* mammary architecture, we assessed the consequences of TC-1 over expression in Matrigel for the three-dimensional morphogenesis assays. Whereas MCF10A cells formed polarized, growth-arrested acinar structures with hollow lumens similar to the glandular architecture *in vivo*, pLenti-TC-1 cells formed small colonies that did not recapitulate this structure. Although the particular morphological phenotype observed in pLenti-TC-1 cells does not indicate exactly how TC-1 transforms cells, it indicates that TC-1 over-expression disrupts epithelial cell architecture, which occurs frequently during the early stages of cancer formation (data not shown). These experiments indicate that TC-1 could induce both IGF-independent growth and anchorage-independent growth, and implies that TC-1 acts as a transforming oncogene when over expressed in breast cancer.

To directly assess the contribution of endogenous TC-1 over expression on the transformation of HBC, we examined the effects of down regulation of endogenous TC-1 expression in SUM-52 and SUM-190 cell lines. As shown in Table I and Figure 1, these two cell lines express TC-1 to the highest levels. First, 2 TC-1 targeted siRNA oligonucleotides, siRNA100 and siRNA175 were introduced into SUM-52 and SUM-190 cells and *TC-1* mRNA levels were subsequently measured by Northern blot. A significant reduction in TC-1 mRNA expression levels was observed 2 days after the TC-1 siRNA treatment. The more efficient siRNA for knockdown of TC-1 was siRNA175 as shown in Figure 3*a*. Thus we chose siRNA175 for both the cell proliferation assays and the anchorage-independent growth assays. To test the effect of siRNA175 on the level of TC-1 mRNA with respect to time, we also performed quantitative real-time PCR analysis in SUM-52 cells treated with siRNA 175 at Days 2, 5 and 9. Efficient knockdown (>80%) was achieved at Day 2, and ~50% at Day 5 and ~25% at Day 9. The consequences of decreased TC-1 expression on growth rates and anchorage-independent growth were evaluated. Figure 3*b* shows that down regulation of TC-1 mRNA suppressed proliferation of SUM-52 and SUM-190 cells. In addition, TC-1 down-regulation mediated by TC-1 siRNA inhibited colony formation significantly in SUM-52 cells compared to control siRNA (Fig. 3*c*). Taken together with the analysis of TC-1 over expressing cells, these results are consistent with a transforming function for TC-1 when it is over expressed in HBC.

F3

### *TC-1 is a downstream target of FGFR2 pathway activation in SUM-52 cells*

A prominent feature of SUM-52 cells is the gene amplification-induced over expression of FGFR2 mRNA and protein. The transforming properties of FGFR2 over expression have been directly demonstrated by treatment of SUM-52 cells with an FGFR-specific small molecule tyrosine kinase inhibitor, PD173074.[16–18] When the PD173074 was used to treat SUM-52 cells, cell proliferation in monolayer culture and colony formation in soft agar were completely inhibited.[16,18] In an effort to determine which genes might be down regulated by this inhibitor and involved in FGFR2 pathways, we performed expression profiling using a 20,000 element cDNA array in SUM-52 cells after exposure to PD173074. Table II lists 18 genes that displayed a >4-fold decrease in expression after treatment with PD173074 in SUM-52 cells (see supplementary data for a list of all genes with >2-fold decrease in expression level). Examination of the expression profile revealed that *TC-1* mRNA levels were dramatically reduced after exposure to PD173074 in SUM-52 cells (Table II). This result was corroborated by northern blot hybridization as shown in Figure 4*a*. To exclude the possibility that TC-1 expression was simply responding to nonspecific mitogenic signaling, we investigated TC-1 expression in SUM190 cells, which harbor HER-2 amplification and over express HER-2 at the message and protein level. TC-1 mRNA expression was not affected by treatment with PD173074 or treatment with the pan-ErbB kinase inhibitor CI-1033 in these cells (Fig. 4*b*) indicating that TC-1 over expression in not a generalized response to mitogenic signaling. Taken together, these data

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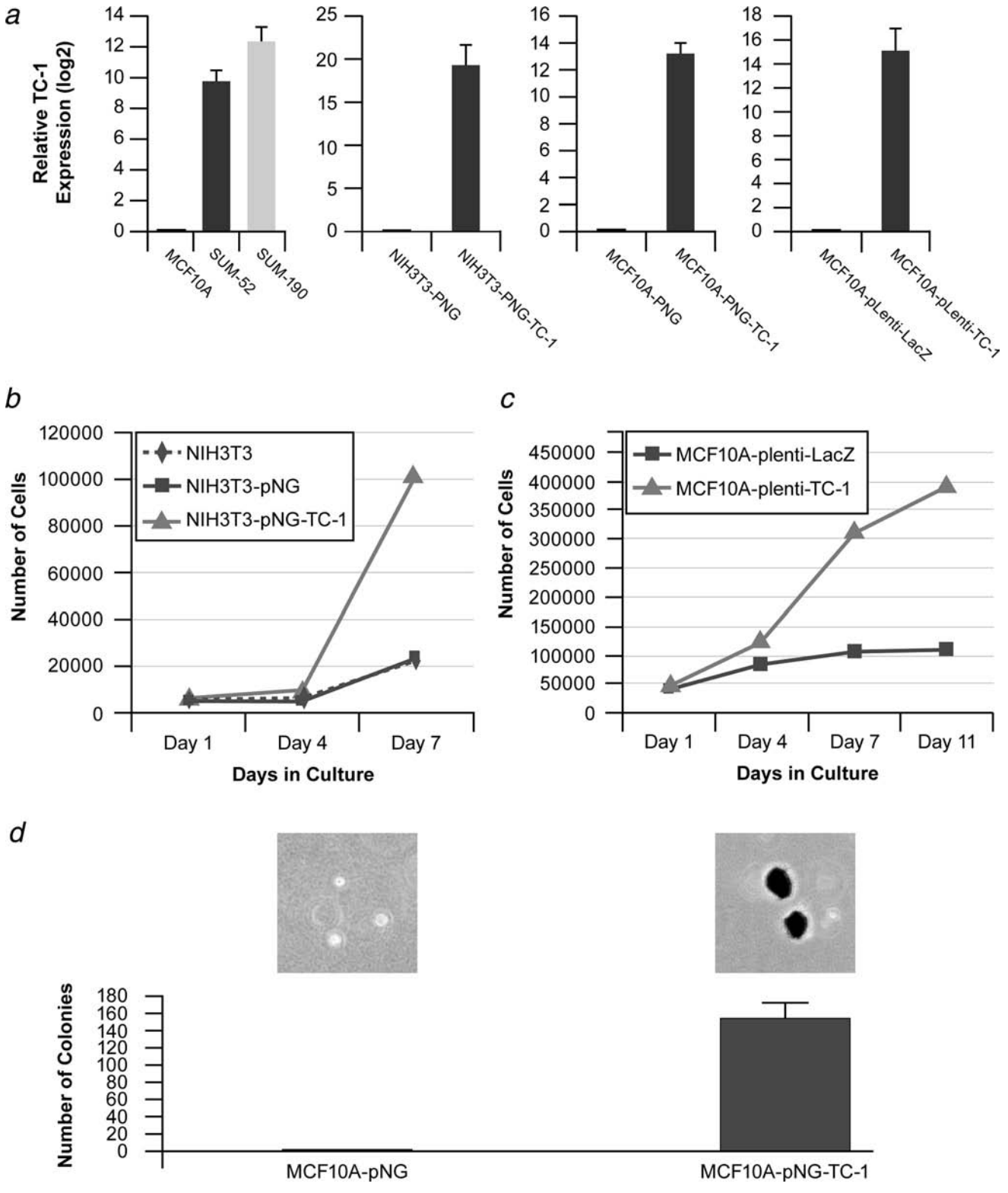


**FIGURE 2** – (*a*) The mRNA expression level of *TC-1* in 2 breast cancer cell lines: SUM-52 and SUM-190, and 3 uncloned cell lines: NIH3T3-pNG-TC-1, MCF10A-pNG-TC-1, and MCF10A-pLenti-TC-1. MCF10A-pLenti-TC-1 cells grow in the absence of insulin-like growth factor medium. *TC-1* expression level (log 2 Ratio) in SUM-52 and SUM-190 relative to control MCF10A cells; in NIH3T3-pNG-TC-1 and MCF10A-pNG-TC-1 relative to their empty vector control cells and MCF10A-pLenti-TC-1 cells relative to MCF10A-pLenti-LacZ control cells were measured by Q-RT-PCR and normalized to GAPDH expression. Briefly, the number of cycles to threshold values for TC-1 were normalized to values for *GAPDH* and then compared to TC-1 expression in control cells to determine their log 2 ratios. For the control cells, the baseline was set to zero. (*b*) *In vitro* growth rate of NIH3T3-pNG-TC-1 that stably over express TC-1 relative to the parental cells or cells containing empty vector, respectively. The cells were seeded in 6-well plates at $3.5 \times 10^3$ cells/well in appropriate growth media. Cell number was determined by counting isolated nuclei with a Coulter counter on days 1, 3, 7 and 11 after seeding. (*c*) *In vitro* growth rate of MCF10A-pLenti-TC-1 cells that stably over express TC-1 relative to MCF10A-LacZ cells in insulin deficient media. Cells were seeded into 35-mm culture wells and grown in the absence of insulin-like growth factors medium. At Days 1, 4, 7 and 11, nuclei were isolated from harvested cells and enumerated with a Coulter counter. (*d*) Number and representative pictures of soft-agar colonies in pNG empty vector infected cells and cells stably expressing TC-1. MCF10A-pNG-TC-1 and pNG-vector cells were grown for 3–4 weeks in soft agar and stained with the vital dye *p*-iodonitrotetrazolium violet.

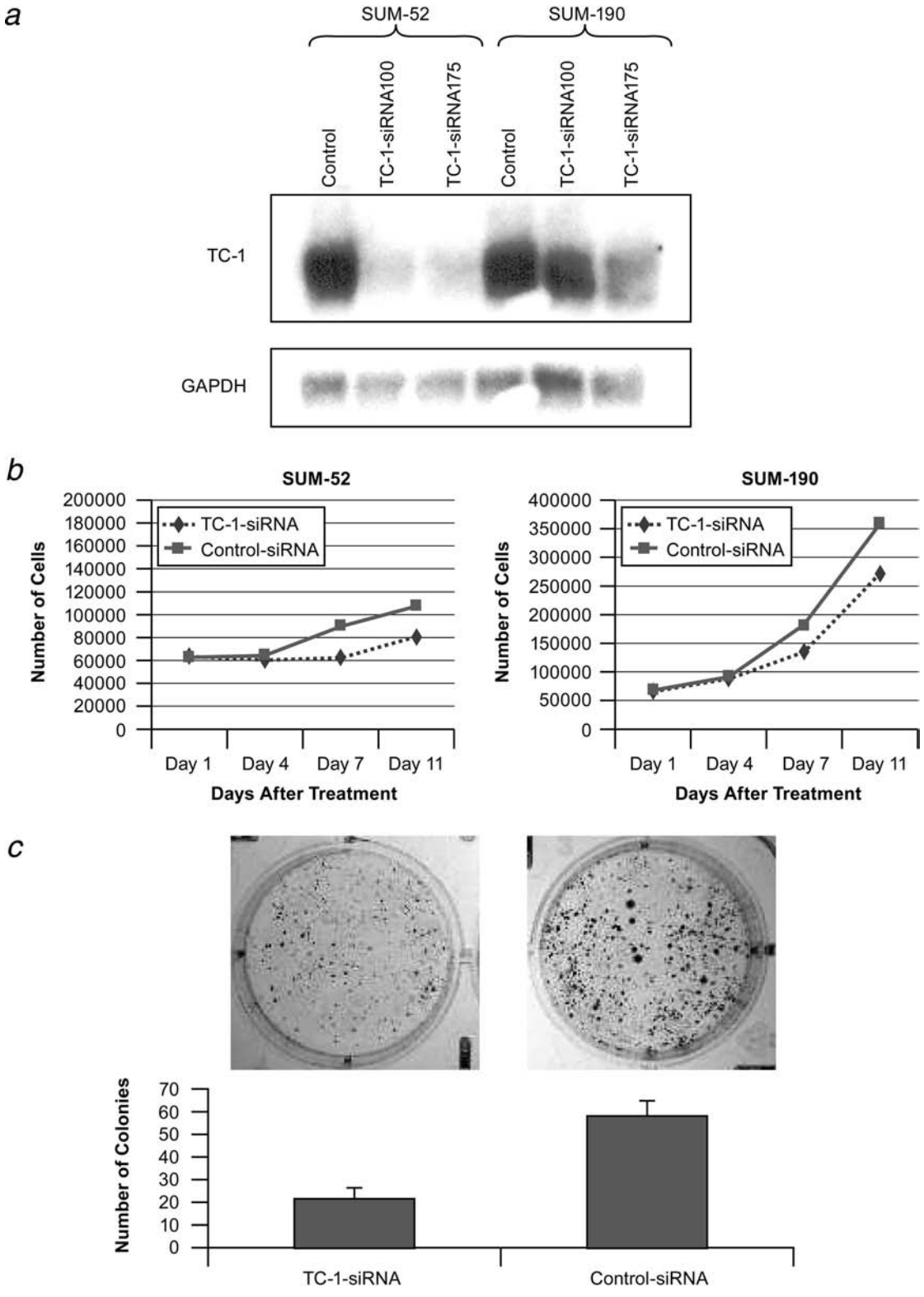


**FIGURE 3** – (*a*) Specific inhibition of TC-1 expression by siRNA in SUM-52 and -190 cells. Two oligonucleotides TC-1-siRNA100 and TC-1-siRNA175 corresponding to human TC-1 sequences starting at the 100th and 175th base in the coding sequence, respectively were synthesized. SUM-52 and SUM-190 cells were transfected with control siRNA oligonucleotides and oligonucleotides specific for TC-1 mRNA. Total RNA was prepared 48 hr after transfection and *TC-1* expression levels were detected by Northern blot. (*b*) *In vitro* growth rate of SUM-52 and SUM-190 cells with TC-1 siRNA treatment compared to cells with control siRNA treatment. (*c*) Knockdown of TC-1 by RNAi reduces colony formation in soft agar. SUM-52 cells were tranfected with TC-1 siRNA or control siRNA. The colony numbers were counted 3 weeks later. A representative well demonstrated the total number of colonies formed by SUM-52 transfected with the indicated siRNA oligonucleotides. The data shown are means and SD from two independent experiments performed in triplicate.

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demonstrate that FGFR2 signaling is involved in the regulation of TC-1 expression.

To confirm that FGFR2 is directly involved in the regulation of TC-1 gene expression, we examined *TC-1* expression levels by Q-RT-PCR in 2 HME cell lines stably over expressing FGFR2. Previously, we identified and isolated 9 different alternatively spliced FGFR2 variants from SUM-52 cells.[16–18] Two variants, FGFR2-C1 and FGFR2-C3, which differ only in their carboxyl termini, have been ectopically expressed in the immortalized HME cell line H16N2.[16,18] *TC-1* transcripts were more than 2-fold up regulated in H16N2 cells stably expressing FGFR2-C1 and C3 compared to cells infected with empty vector alone (Fig. 4*c*). Thus, increasing the level of FGFR2 coincides with induction of endogenous *TC-1* mRNA, lending additional support to the notion of FGFR2 as a regulator of TC-1 expression as observed in SUM-52 cells.

**TABLE II** – DOWN-REGULATED GENES FOLLOWING EXPOSURE TO PD173074 IN SUM-52 CELLS

| Gene symbol | Unigene | Fold |
|---|---|---|
| *EST* | AA609920 | 8.06 |
| *CAMSAP1* | AA176911 | 7.33 |
| *C8orf4 (TC-1)* | H16793 | 6.68 |
| *MAN1A2* | AA455062 | 6.04 |
| *EST* | W95948 | 5.35 |
| *HSA9761* | AA418523 | 5.08 |
| *MTRF1L* | R44397 | 5.01 |
| *FOXM1* | AA129552 | 4.93 |
| *DDIT4* | H92504 | 4.85 |
| *SBBI81* | AA172096 | 4.79 |
| *PLOD2* | H99816 | 4.64 |
| *FLJ14054* | N90806 | 4.55 |
| *GCSH* | R28294 | 4.48 |
| *SLC7A1* | R28280 | 4.46 |
| *EPHB2* | W96473 | 4.27 |
| *FAM46C* | AA058597 | 4.24 |
| *EST* | N63114 | 4.23 |
| *FH* | AA026917 | 4.12 |

### *Global expression of β-catenin target genes are not positively associated with TC-1 expression in breast cancer*

Since TC-1 has been implicated as a modulator of Wnt/β-catenin signaling, we tested whether TC-1 over expression is associated with an increased expression of β-catenin target genes in our 11 SUM-breast cancer cell lines, MCF10A-pNG-TC-1 cells and MCF10A cells. Gene expression profiles were obtained using Affymetrix U133A arrays, which include 122 probe sets for 58 Wnt/β-catenin pathway target genes. These target genes were identified from a review of the literature, and from those listed on the Nusse laboratory website (http://www.stanford.edu/~rnusse/wntwindow.html). Probe sets measuring expression for a given

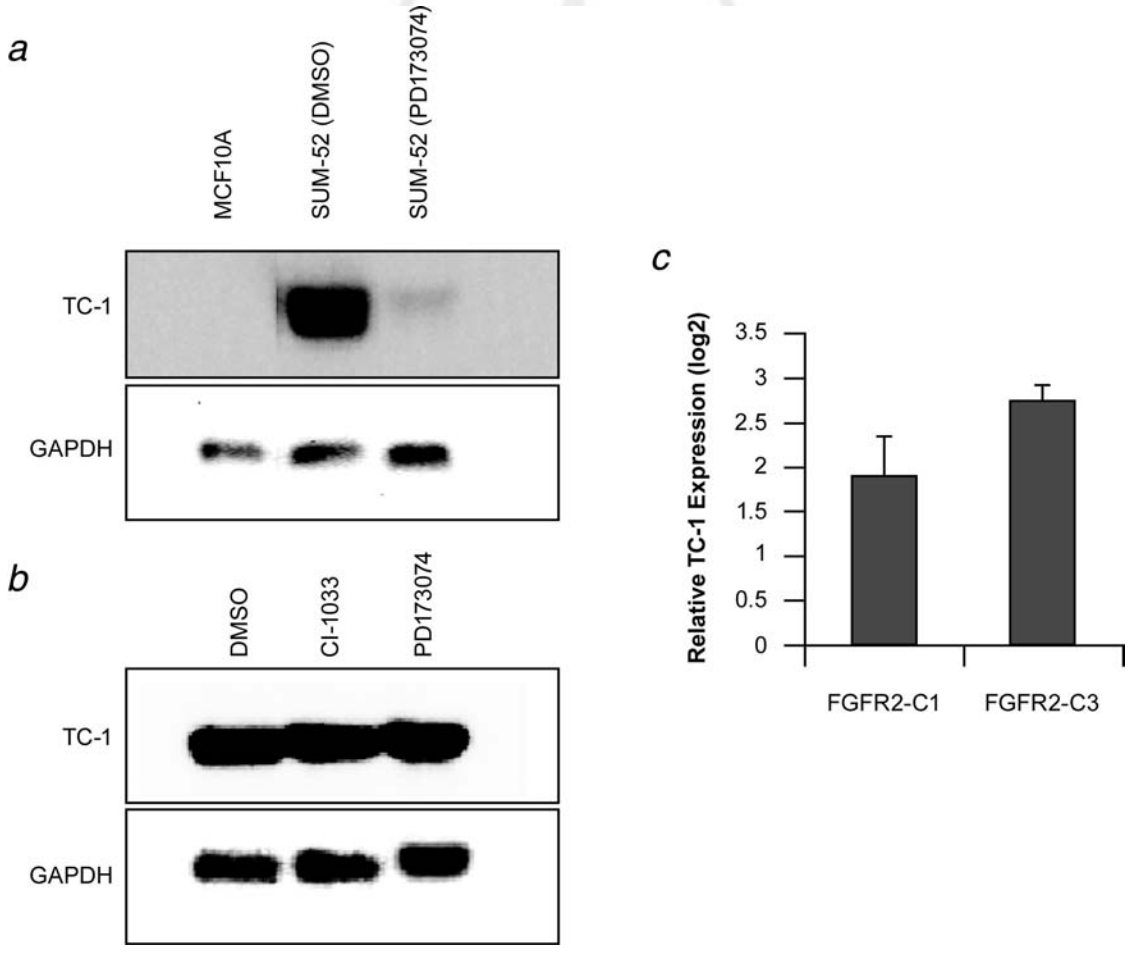


**FIGURE 4** – (*a*) Northern blot analysis shows *TC-1* expression is decreased after the FGFR inhibitor PD173074 treatment in the SUM-52 breast cancer cell line. Cells were treated with 1uM PD173037 for 24 hr. (*b*) Northern blot analysis shows *TC-1* expression was not affected by treatment with PD173074 or treatment with the ErbB specific kinase inhibitor CI-1033 in SUM-190 cells. (*c*) Relative *TC-1* mRNA levels were measured by Q-RT-PCR in H16N2-FGFR2-C1 and H16N2-FGFR2-C3 cells. TC-1 expression level (log 2 ratio) relative to H16N2-Vector cells was normalized to GAPDH expression.

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transcript were averaged and then used as parameters to generate a generalized linear model with *TC-1* expression as a logistic response.[30] This test can find significant associations between TC-1 expression and many subtle gene expression changes. When considered together in this way, the gene expression patterns of these 58 genes did not significantly associate with *TC-1* gene expression levels ($p < 0.075$).

The 41 Wnt/β-catenin target genes whose average expression was greater than 8 intensity units were subjected to hierarchical clustering (Fig. 5*a*). Clearly, no discernable overall pattern of expression relative to *TC-1* expression was evident (Fig. 5*a*). Nevertheless, several individual β-catenin target genes were found to be highly expressed in all but 1 breast cancer cell line that exhibited TC-1 over expression, including *VEGF*, *CLDN1* and *CDH1* (Fig. 5*a*). Up-regulation of *CLDN1* and *CCND1* in TC-1 over expressing cells was verified by Q-RT-PCR analysis (Fig. 5*b*). In addition, there was a subcluster of genes whose expression was inversely correlated with TC-1 expression (Fig. 5*a*). Interestingly, both MCF10A-TC-1 cells and SUM-206 cells have high and medium levels of TC-1 expression, but do not cluster with the other TC-1 over expressing cells. Finally, we did not observe nuclear β-catenin in SUM-52 cells, nor do these cells exhibit any detectable TCF/LEF transcriptional activity (data not shown). The mechanisms controlling expression of β-catenin target genes are complex and involve other signaling pathways and transcription regulatory factors. Given that there was no association of TC-1 over expression with global expression of β-catenin target genes, and the lack of direct evidence for β-catenin signaling in SUM-52 cells, the high expression of some β-catenin target genes observed in our experiments likely occurs independently of β-catenin signaling, but in a manner that is regulated by TC-1.

Discriminant analysis was also undertaken to determine if there were any gene expression differences between TC-1 over expressing cells and TC-1 nonexpressing cells. This analysis revealed only 1 gene whose expression differed significantly between the groups, TC-1 itself ($p < 0.00039$, fdr corrected).

## Discussion

In this investigation, we have found that TC-1 is a candidate oncogene in breast cancer. Over expression of *TC-1* mRNA occurred in ~50% of breast cancer cell lines and primary breast cancers.[31] The role of TC-1 in the growth of breast cancer was evaluated in several experimental models. Its forced expression in MCF10A cells stimulated both their insulin-independent growth and anchorage-independent growth in soft agar. Furthermore, RNAi-mediated knock-down of TC-1 resulted in the loss of anchorage-independent growth capacity in SUM-52 breast cancer cells. Together, these data suggest that TC-1 is involved in tumor progression of a subset of breast cancer.

Recent studies have begun to shed light on the tumorigenic role of TC-1 in thyroid and gastric cancers.[8,9,19] TC-1 is implicated in thyroid cancer by virtue of its over expression in more than 90% of cases.[8] Forced expression of TC-1 in normal thyroid cells increased their proliferation rate and enhanced their anchorage-independent growth in soft agar.[10] In gastric cancer, TC-1 expression correlated strongly with nearly all pathologic variables of aggressive biological behavior. RNAi-mediated TC-1 knock-down showed significant inhibition of proliferation in the gastric cell line KATO-III.[9,19] The ability of TC-1 to induce cell transformation in our model is consistent with a potential tumorigenic function in thyroid and gastric cancers.

An important finding of our study is that TC-1 is linked to FGFR2 signaling pathway in breast cancers, in which the gene is amplified and over expressed. Over expression of FGFR's occurs frequently in numerous types of human cancer, including gastric, breast and thyroid cancers and correlates with poor prognosis and shorter survival time.[14,15] *FGFR2* was first discovered as an amplified gene in the gastric cancer cell line KATO-III and over

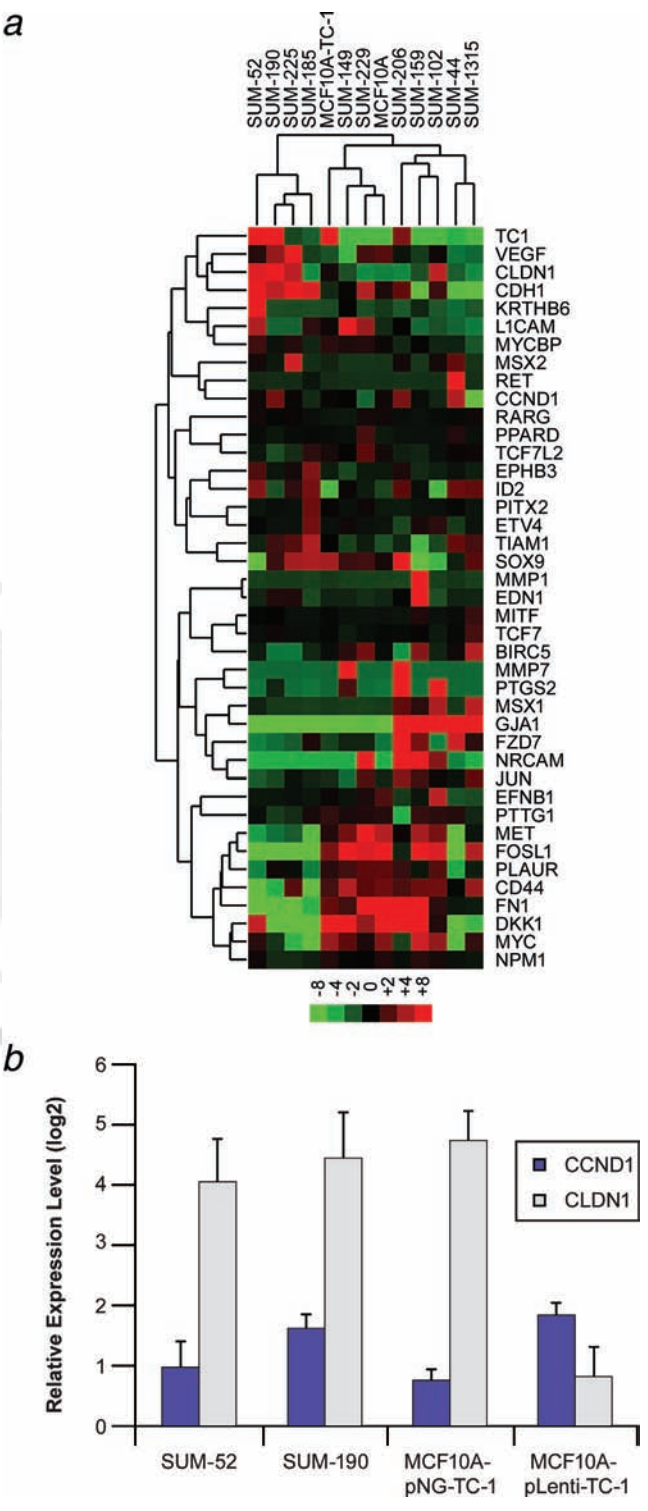


FIGURE 5 – (*a*) Unsupervised hierarchical clustering of *TC-1* and 41 Wnt/β-catenin target genes in our 11 SUM-breast cancer cell lines, MCF10A cells and MCF10A-TC-1 cells. Each row represents a target gene, and each column represents a cell line. Color codes are indicated at the bottom of the figure. (*b*) Two β-catenin target genes, *CCND1*, and *CLDN1* mRNA levels were measured by Q-RT-PCR in: SUM-52 and SUM-190 cells relative to MCF10A, MCF10A-pNG-TC-1 cells relative to empty vector cells and MCF10A-pLenti-TC-1 cells relative to pLenti-LacZ cells.

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expression of FGFR2 plays a pivotal role in gastric cancer development.[32,33] Interestingly, as indicated earlier, KATO-III cells also have high level over expression of TC-1, which plays a role in the growth potential of these cells.[9] Previously, we demonstrated that SUM-52 cells have an *FGFR2* gene amplification and dramatically over expresses FGFR2 at both the message and the protein levels.[17] Thus, SUM-52 cells have 2 amplified regions harboring oncogenes that are mechanistically linked. The 10q26 region harbors *FGFR2*, and the 8p11-p12 region harbors *TC-1*. In the present study, we demonstrated that FGFR2 expression regulates the expression of the coamplified TC-1. We have recently identified and isolated 9 alternatively spliced FGFR2 variants from SUM-52 cells. Among these isoforms are 2 otherwise identical FGFR2 variants that express either the C1 or C3 carboxyl terminus.[16–18] Here we showed that ectopic FGFR2-C1 and C3 expression are both able to enhance TC-1 mRNA expression in H16N2 cells. However, FGFR2-C3 cells have higher levels of TC-1 expression compared to FGFR2-C1 cells. Our previous results and others demonstrated that expression of FGFR2-C3 leads to more potent transformation than FGFR2-C1.[16,18,34] Thus TC-1 over expression is mechanistically linked to FGFR2 pathways and may be involved in the transforming properties of FGFR2 splicing isoforms in cancer.

Recently, it was found that TC-1 is a positive regulator of the Wnt/β-catenin pathway in gastric cancer. TC-1 up-regulates β-catenin target genes through interaction with Cby in HEK293T cells and in the gastric cancer cell line KATO-III.[9,19] Cby is an endogenous inhibitor of the canonical Wnt signaling pathway. Cby binds to the LEF/TCF interaction domain at the C-terminus of β-catenin and thereby eliminates interaction of β-catenin with LEF/TCF transcription factors.[20] This leads to a block of transcriptional activation within the signaling cascade. TC-1 competes with β-catenin for the interaction with Cby, and thereby enhances the signaling pathway through relieving the suppression by Cby. However, the gene expression patterns of 58 β-catenin target genes did not significantly associate with TC-1 gene expression levels in either our breast cancer cell lines or TC-1 over expressing MCF10A cells. Furthermore, we found no evidence for nuclear β-catenin in SUM-52 cells. In addition, TOP/FOPFLASH reporter assays using the TCF/LEF reporter construct showed no evidence for β-catenin biological activity in SUM-52 cells (data not shown). Nevertheless, we did find several Wnt/β-catenin target genes, including *CLDN1* and *CCND1* that were highly expressed in cell lines that exhibited TC-1 over expression. Koziczak *et al*. and our present study (data not shown) also demonstrated that treatment of SUM-52 cells with the FGFR-selective inhibitor, PD173074, caused down regulation of the β-catenin target CCND1. However, the mechanisms controlling expression of β-catenin target genes are complex and do not depend solely on β-catenin/TCF/LEF activity.[21,35–37] Furthermore, there are a number of other factors besides Cby that bind to the TCF–β-catenin complex and are necessary for transcriptional activation, including legless (lgs) and Pygopus (Pygo).[38–41] Different cell types may respond to Wnt signaling by transcribing different sets of target genes. Clearly, additional work will be required to precisely define whether TC-1 participates with the Wnt/β-catenin pathway in the development and progression of breast cancer.

The high frequency of TC-1 over expression in breast cancer suggests TC-1 may be regulated by pathways other than FGFR2. Friedman *et al*. found that TC-1 was a TGF-β-induced gene in colon cancer cell lines.[42] In addition, TC-1 is up-regulated by IL-1β and TNF-α and is inhibited by IKK/NF-κB inhibitors in human follicular dendritic cells.[43] We also found that TC-1 is up-regulated by IL-1 in normal mammary epithelial cells (data not shown).

In summary, our study revealed that TC-1 is a transforming gene when over expressed in HBC cells. In some cases, TC-1 is downstream of FGFR2 mediated signaling pathways. TC-1 over expression is also associated with expression of some β-catenin target genes, which may play an important role in mediating the expression of transformed phenotypes. As a novel regulator of these pathways, TC-1 may be implicated in regulating the biological behavior of breast cancer through coordinated activation of key signaling molecules known to play a role in cell transformation.

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# Multiple Interacting Oncogenes on the 8p11-p12 Amplicon in Human Breast Cancer

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## Abstract

**The 8p11-p12 genomic region is amplified in 15% of breast cancers and harbors several candidate oncogenes. However, functional evidence for a transforming role for these genes is lacking. We identified 21 genes from this region as potential oncogenes based on statistical association between copy number and expression. We further showed that three of these genes (*LSM1*, *BAG4*, and *C8orf4*) induce transformed phenotypes when overexpressed in MCF-10A cells, and overexpression of these genes in combination influences the growth factor independence phenotype and the ability of the cells to grow under anchorage-independent conditions. Thus, *LSM1, BAG4*, and *C8orf4* are breast cancer oncogenes that can work in combination to influence the transformed phenotype in human mammary epithelial cells.** (Cancer Res 2006; 66(24): 11632-43)

## Introduction

One of the main molecular pathways in the natural history of human breast cancer development involves the focal amplification of distinct regions of the genome, resulting in copy number and expression changes of specific genes within these regions (1). Thus, gene amplification and overexpression is a major mechanism for oncogene activation in human solid cancers like breast cancers (1–6). Although the genetic mechanisms that result in copy number increases occur over the entire genome, selection of cells with genomic alterations in specific regions occurs in a non-random fashion during the progression of breast cancer, supporting the hypothesis that these regions harbor dominantly acting oncogenes that play a causal role in cancer progression. The 17q21 genomic region is one of the most well studied regions of gene amplification in breast cancer because this amplicon harbors the *ERBB2* oncogene, along with other important genes, such as *GRB7* and *TOPO2A* (7–11). Other important regions in breast cancer include the 8q24 amplicon, (12–15), the 20q13 amplicon (16–19), and the 11q12 amplicon (20–25).

We have recently developed a novel panel of human breast cancer cell lines derived from several different molecular subclasses of human breast cancer (2, 26). Previous global genome analysis studies on these cell lines revealed that three cell lines in the panel (SUM-44, SUM-52, and SUM-225) harbor focal copy number increases in the 8p11-12 region (2, 3). Furthermore, past and recent studies on primary breast cancer specimens have shown that gene amplification occurs in this region in 10% to 15% of human breast cancers (14, 27–31). Recent studies from our lab and others have suggested that the heretofore best candidate oncogene from this region (*FGFR1*) is not the only candidate oncogene in the region and, indeed, may be of marginal significance (32). Additionally, other candidate oncogenes have emerged based on statistical analysis of associated copy number changes and expression levels of genes in this region.

In the present studies, we sought to extend the correlative analysis of copy number and expression level of genes on the 8p11-p12 amplicon in human breast cancer specimens and cell lines by examining the amplicon in a panel of primary human breast cancers with copy number increases in this region. In addition, we used quantitative reverse transcription-PCR (RT-PCR) to determine the expression level of 53 genes from this region in breast cancer specimens compared with three different types of normal human mammary epithelial cells. To move beyond correlative approaches, we employed an alternative strategy aimed at identifying candidate oncogenes directly based on their ability to transform the immortalized human mammary epithelial cell line MCF-10A to specific growth factor independence. From these experiments, we have shown that *LSM1, BAG4*, and *C8orf4(TC-1)* are bona fide breast cancer oncogenes based on their copy number and expression status in human breast cancer and their ability to transform human mammary epithelial cells *in vitro*. In addition, these genes can cooperate to influence the expression of important altered growth phenotypes, which supports the hypothesis that common amplicons that occur in breast and other cancers harbor multiple oncogenes that can cooperate to influence the growth potential of cancer cells.

## Materials and Methods

**Cell lines and tumor specimens.** The isolation and culture of the SUM series of HBC cell lines and MCF10A cells have been described in detail previously (2, 3). Human breast cancer specimens were obtained from Asterand, Inc. (Detroit, MI).

**Quantitative genomic PCR.** Quantitative genomic PCR experiments used the Applied Biosystems Prism 7900HT Sequence Detection System. The *ZNF703(FLJ14299), LSM1, FGFR1, C8orf4*, and *GAPDH* Taqman probe and primer mix as well as the Taqman Universal PCR master mix were purchased from Applied Biosystems (Foster City, CA). The data were analyzed using Sequence Detector System v2.1 (Applied Biosystems) and Microsoft Excel software.

**Genomic array comparative genomic hybridization.** Genomic array comparative genomic hybridization (CGH) experiments were done using the Agilent 44K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA). The protocol used for this experiment was The Oligonucleotide Array-Based CGH for Genomic DNA Analysis (Agilent Technologies). For each array, female DNA (Promega, Madison, WI) was used as a reference sample and labeled with Cy-3. The biological samples of interest were each labeled with Cy-5.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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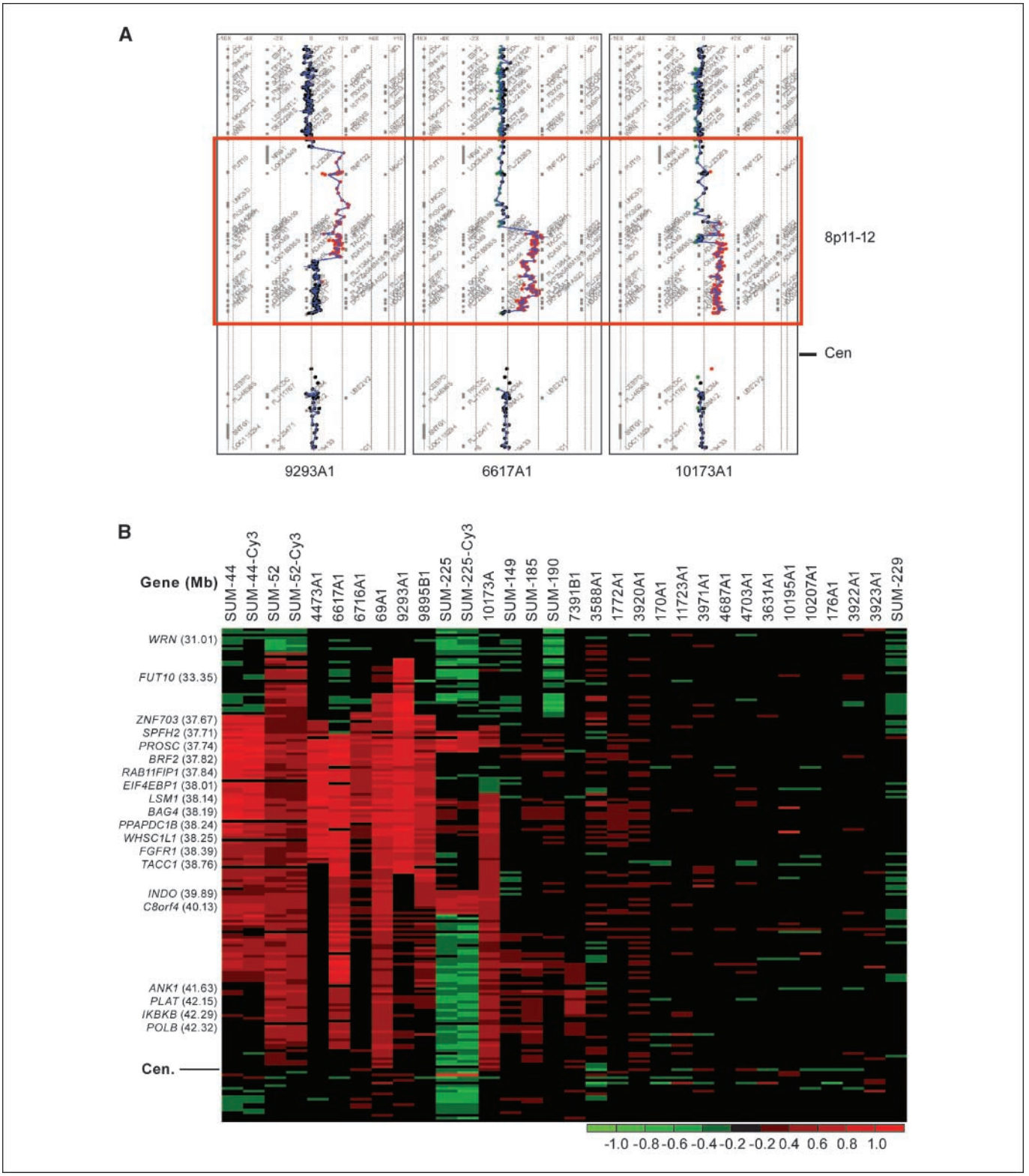
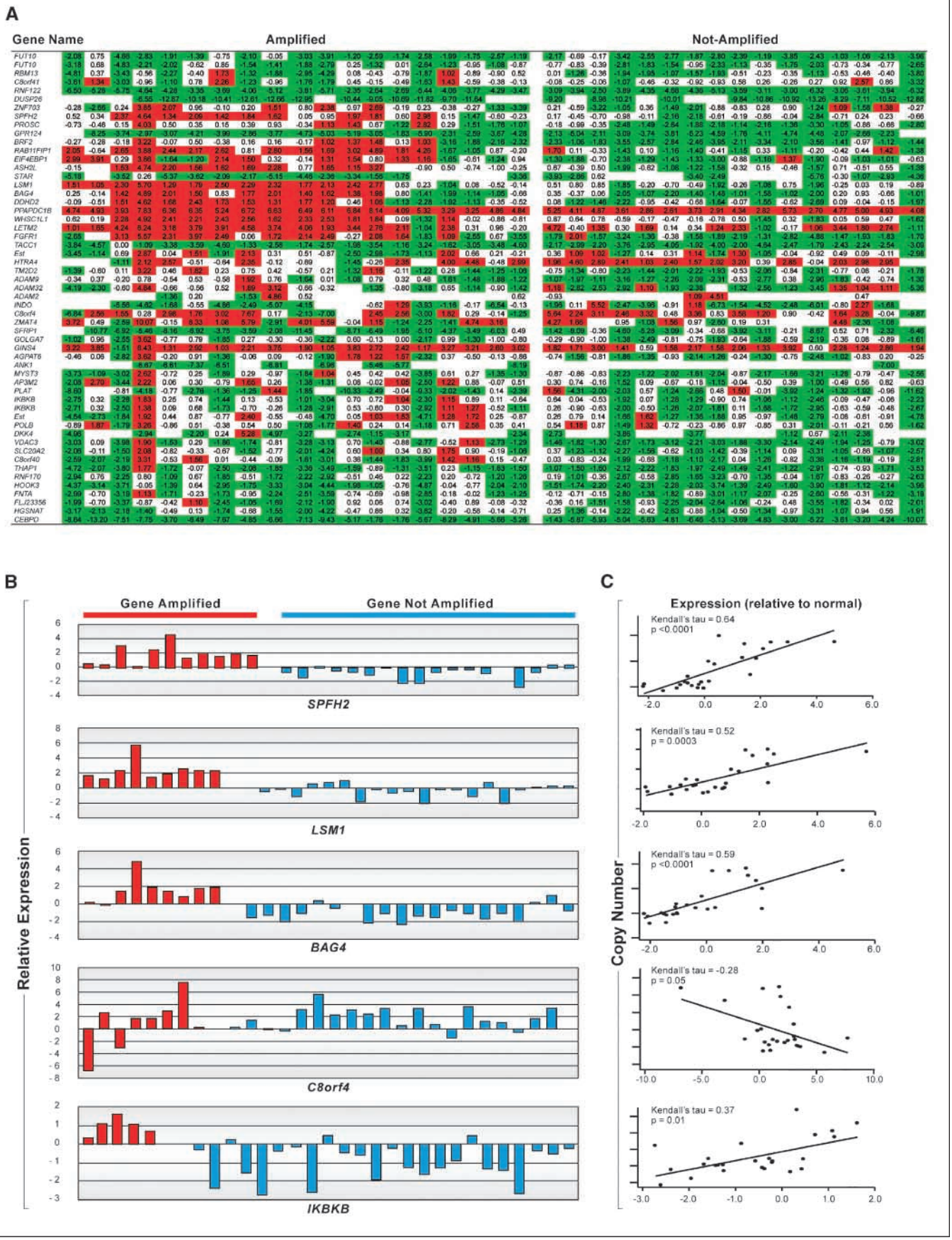



**Figure 1.** Array CGH analysis of the 8p11-p12 region in 7 human breast cancer cell lines and 22 primary breast cancer specimens. *A,* genome view of the 8p11-p12 amplicon region analyzed on the Agilent oligonucleotide array (Agilent Technologies) in three primary breast cancer specimens 9293A1, 6617A1, and 10173A1. Genes are arranged in genomic order from 8pter to 8qter. Results are visualized in CGH Analytics (Agilent Technologies). *B,* genomic copy number profiles of the 8p11-p12 amplicon region analyzed on the Agilent oligonucleotide array CGH in 7 SUM breast cancer cell lines and 22 primary breast cancer specimens. Tumors are displayed vertically, and array probes are displayed horizontally by genome position. Log 2 ratio in a single sample is relative to normal female DNA and is depicted according to the color scale (*bottom*). *Red,* relative copy number gain; *green,* relative copy number loss. Three cell lines (SUM-44, SUM-52, and SUM-225) have duplicated data sets. Based on the array CGH data, there appears to have been a significant false-positive rate of detection of copy number increase using the quantitative genomic PCR screen. Most of the differences occurred in specimens in which only the *ZNF703* locus showed evidence of copy number increase. At the present time, the basis for the difference in results between the quantitative genomic PCR screen and the array CGH analysis is not known.


A
Gene Name
Amplified
Not-Amplified
FUT10
FUT10
RBM13
C8orf41
RNF122
DUSP26
ZNF703
SPFH2
PROSC
GPR124
BRF2
RAB11FIP1
EIF4EBP1
ASH2L
STAR
LSM1
BAG4
DDHD2
PPAPDC1B
WHSC1L1
LETM2
FGFR1
TACC1
Est
HTRA4
TM2D2
ADAM9
ADAM32
ADAM2
INDO
C8orf4
ZMAT4
SFRP1
GOLGA7
GINS4
AGPAT6
ANK1
MYST3
AP3M2
PLAT
IKBKB
IKBKB
Est
POLB
DKK4
VDAC3
SLC20A2
C8orf40
THAP1
RNF170
HOOK3
FNTA
FLJ23356
HGSNAT
CEBPD
B
Gene Amplified
Gene Not Amplified
Relative Expression
SPFH2
LSM1
BAG4
C8orf4
IKBKB
C
Expression (relative to normal)
Copy Number
Kendall's tau = 0.64
p <0.0001
Kendall's tau = 0.52
p = 0.0003
Kendall's tau = 0.59
p <0.0001
Kendall's tau = -0.28
p = 0.05
Kendall's tau = 0.37
p = 0.01

Agilent's CGH Analytics software was used to calculate various measurement variables, including log 2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe. Chromosomal aberrations were classified as amplification when the log 2 ratio was >0.33 and as loss when the ratio was less than −0.33. This number was determined based on the array data and our previous fluorescence *in situ* hybridization and Southern data in three cell lines.

**Microfluidics-based quantitative RT-PCR.** Predesigned Taqman probe and primer sets for 8p11-12 target genes and housekeeping genes were chosen, factory-loaded into the 384-well format, and spotted on a microfluidic card by the manufacturer ( four replicates per assay). RNA was isolated from breast cancer specimens and cell lines as well as three different types of normal human mammary epithelial cells (normal breast tissue, MCF-10A cells, and HME cells) and converted into cDNA. Quantitative real-time PCR was done in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Gene expression values were calculated based on the $\Delta\Delta C_t$ method. The expression levels of the housekeeping gene *GUSB* from each sample were used for normalization because this gene showed relatively constant expression across most of the analyzed samples.

**Production of lentivirus and cell infection.** Lentiviral expression constructs were created for candidate oncogenes from the 8p11-p12 amplicon, including *LSM1, EIF4EBP1, C8orf4, PPAPDC1B(HTPAP), ZNF703, RAB11FIP1, BAG4*, and *FGFR1*, using ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA). Each construct was sequenced to ensure that the sequences and orientation are correct. Lentivirus was produced by cotransfecting the 293FT cell line with the pLenti expression construct and the optimized packaging mix (Invitrogen). MCF-10A cells were transduced with lentivirus, and gene expression level was detected using RT-PCR. For combination infections, virus of the same titer from two, three, or all eight genes was combined equally and used to infect MCF-10A human mammary epithelial cells. Control infections with pLenti-LacZ virus were done in parallel with other infections. Selection began 48 hours after infection in growth medium with 10 μg/mL blasticidin and without either insulin or epidermal growth factor (EGF). Upon confluence, selected cells were passaged and serially cultured.

**Growth in soft agar.** Soft agar assays were done as previously described (33). Briefly, dishes were coated with a 1:1 mix of the appropriate 2× medium for the cell line being studied and 1% Bactoagar. Cells were plated at $1 \times 10^5$ per well, fed thrice per week for 3 to 4 weeks, stained with 500 μg/mL *p*-iodonitrotetrazolium violet (Sigma, St. Louis, MO) overnight, and counted.

**Immunoprecipitation and Western blots.** Cell lysis and protein quantification were done as previously described (34). For immunoprecipitations, whole-cell lysate (1 mg) was incubated with 2 μg/mg IRS-1 antibody for 2 hours at 4°C or 2 μg/mg EGF receptor (EGFR) antibody for 1 hour on ice followed by incubation with protein A/G agarose beads (Sigma) for 1 hour at 4°C. Proteins were resolved on 7.5% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed for 1 hour at room temperature with 2 μg/mL IRS-1 (Upstate, Lake Placid, NY), p-Tyr (PY20; BioMol International, Plymouth Meeting, PA), or EGFR (Zymed, South San Francisco, CA) antibodies. Membranes were incubated for 1 hour at room temperature in peroxidase-labeled secondary antibody and developed in enhanced chemiluminescence (Pierce, Rockford, IL).

**Assay for monolayer growth and conditioned medium activity in insulin-independent and EGF-independent clones.** For growth experiments, insulin-independent, EGF-independent, and control cells were seeded into six-well plates at $3.5 \times 10^4$ per well and grown in their appropriate medium. The EGF-independent clones were treated with 0.1 or 0.5 μmol/L of the EGFR inhibitor Iressa every 24 hours. Cell number was measured on day 9 using a Coulter counter.

Conditioned medium from subconfluent clones of MCF-10A cells infected with all combinations of two or three of *LSM1, BAG4*, and *C8orf4* was collected. MCF-10A cells were seeded in six-well plates and grown in normal growth medium, medium without insulin (SFHE) or EGF (SFIH), or SFHE/SFIH medium supplemented 1:1 with conditioned medium from clones without insulin or EGF. Cell number was measured on day 9 using a Coulter counter.

**Statistical methods.** Kendall's tau was used to assess the statistical significance of the association between copy number and expression for each gene. Holm's stepdown procedure was used to adjust significance levels for the large number of estimates to reduce the likelihood of false positive results. We used $P = 0.01$ as a cutoff for a statistically significant association between copy number and expression.

## Results

Recently, we analyzed the 8p11-p12 genomic region for copy number and gene expression changes in three human breast cancer cell lines developed in our laboratory (32). From these experiments, we provided evidence that *FGFR1* is not the only candidate breast cancer oncogene in this region. To provide further correlative and causal evidence for a transforming function of candidate genes from the region, we examined the 8p11-p12 region in 100 primary breast cancer specimens and cell lines at both the copy number and expression levels. We also analyzed the transforming potential of candidate oncogenes when overexpressed either individually or in specific combinations using a cell transformation assay based on the growth factor dependency of the human mammary epithelial cell line MCF-10A.

**DNA copy number analysis in a panel of primary human breast cancers.** One hundred unselected primary human breast cancers were screened for the presence of copy number increases in the 8p11-p12 region. As a preliminary screen for gene copy number increases in the 8p11-p12 region in these specimens, quantitative genomic PCR was done using primers for *ZNF703, LSM1, FGFR1*, and *C8orf4*, as these four genes are present within the amplified region as defined in the previous work. The results of this analysis showed that, of the 90 breast cancer specimens that yielded usable DNA, 24 tumors showed evidence of copy number increase (log 2 > 2) at one or more of the loci (see Supplementary Table S1). Twenty-two of these specimens yielded RNA of sufficient quality and quantity for expression analysis. In addition, we identified three more breast cancer specimens from our own bank

**Figure 2.** Expression level of genes located at 8p11-p12 and statistical analysis of expression/amplification correlations of five representative genes in 8p11-p12 amplicon. *A,* each row corresponds to one gene probe ordered according to its genomic position from 8pter to 8qter (two probes for FUT10 and IKBKB). Each column represents one primary breast cancer or breast cancer cell line sample. Samples are grouped according to whether they show amplification or no amplification of the 8p11-p12 region based on array CGH and/or quantitative genomic PCR data. Expression level of genes located at 8p11-p12 was detected by quantitative RT-PCR–based microfluidic cards. The log 2 transformed expression level of each gene in a single sample is relative to its abundance in normal breast tissue. *Red,* genes in which the log 2 ratio of expression in the tumor compared with normal tissue was >1.0 (2-fold relative increase in expression). *Green,* genes in which the log 2 ratio of tumor versus normal is less than −1.0. Similar results were obtained when using RNA from either MCF-10A cells or from telomerase immortalized human mammary epithelial cells as controls (see Supplementary Data). *B,* the graphs show the correlation between mRNA expression level and DNA amplification status in 3 human breast cancer cell lines and 26 primary breast cancer specimens. Gene expression levels (log 2) relative to normal human breast tissue were obtained from quantitative RT-PCR after normalization to a housekeeping gene (*GUSB*). Gene amplification status was based on the array CGH data. *C,* statistical analysis of expression/amplification correlations of five representative genes showed that *SPFH2, BAG4, LSM1*, and *IKBKB* mRNA expressions are associated with DNA amplification status, whereas *C8orf4* overexpression is independent of its amplification status in breast cancer specimens.

and two additional breast cancer cell lines with copy number increases in the 8p11-p12 region and included these specimens in further studies.

To obtain a detailed map of the breast cancer specimens with putative copy number changes at 8p11-p12, the 22 breast cancer specimens identified in the preliminary screen were analyzed further by array CGH using the Agilent human genome CGH microarray chip. Representative array CGH profiles of three breast cancer specimens are shown in Fig. 1*A*, and copy number data for all 22 specimens analyzed are shown in Fig. 1*B* (see Supplementary Data for all array CGH profiles). The results of array CGH analysis confirmed and extended the results of previous experiments and showed the variations in amplicon structure within this region in different breast cancer specimens and cell lines. Interestingly, only 11 of the 22 specimens analyzed by array CGH were confirmed to have copy number increases in the 8p11-p12 region. Thus, in our panel of breast cancer specimens, the frequency of copy number increase in the 8p11-p12 region was ~13% (11 of 90), which is consistent with estimates from other laboratories.

Figure 1*B* shows the copy number increases in the 8p11-p12 genomic region in all 22 breast cancer specimens that were analyzed by array CGH, as well as several breast cancer cell lines. Analysis of these results indicates the presence of a core region of gene amplification that spans from *ZNF703* to the *FGFR1* gene in all but one of the breast cancers. In addition, several breast cancers and cell lines contain this core region of amplification in addition to a more centromeric region of copy number increase that is often, but not always, contiguous with the core amplified domain. One breast cancer specimen in our panel (7391B1) showed evidence of copy number increase in the centromeric region that was independent of the core amplified domain. Thus, the results of these experiments indicate that the 8p11-p12 genomic region is frequently amplified in breast cancer and harbors a number of genes that are potentially important in breast cancer progression.

**Expression of genes from the 8p11-p12 region in breast cancers and cell lines with gene copy number increases and controls.** As shown previously, the region of copy number increase in the 8p11-p12 region spans ~10 Mb and encompasses ~53 known genes. To measure the expression level of these 53 genes in our panel of primary breast cancer specimens and breast cancer cell lines, both with and without gene amplification, we designed a microfluidics-based Taqman quantitative RT-PCR assay using primers specific for each of the 53 genes. RNA was isolated from 13 primary breast cancers and five breast cancer cell lines with an 8p11-p12 amplification, from 15 breast cancer specimens and two cell lines lacking the amplicon, and from three different types of normal human mammary epithelial cells. The results of this analysis are presented in Fig. 2*A* and show the expression level of each of the 53 genes in all 35 specimens relative to the expression levels obtained with normal breast tissue RNA. Examination of the data in Fig. 2*A* reveals a set of genes that are selectively overexpressed in the breast cancer specimens and cell lines with gene copy number increases in the 8p11-p12 region. Genes, such as *LSM1, SPFH2*, and *BAG4*, were both amplified and overexpressed in a significant fraction of the tumors analyzed, and expression levels for these genes were low when the genes were not amplified. Similar results were obtained for genes, such as *IKBKB, POLB*, and *VDAC3*, but the number of tumors with amplification and overexpression of these genes was smaller than those within the core amplified domain. In addition, genes, such as *C8orf4*, were overexpressed in breast cancer specimens independent of their copy number status.

To examine the relationship between copy number status and expression for all genes in the amplified region in all of the specimens in the panel, Kendall's tau, a measure of association, was used to assess the statistical significance of the association between copy number and expression for each gene. Holm's stepdown procedure was used to adjust significance levels for the large number of estimates, thereby reducing the likelihood of false-positive results. Figure 2*B* shows the expression level of a subset of genes from the region in breast cancer specimens both with and without copy number increases. Figure 2*C* shows the statistical relationship between copy number and expression for the same subset of genes for all of the breast cancer specimens and cell lines analyzed. The data show that the expression of some genes is tightly linked to their copy number status, whereas for some genes, there is no relationship between expression and copy number. Table 1 shows the statistical analysis of copy number and expression for all 53 genes in the region. Using $P = 0.01$ as a cutoff for a statistically significant association between copy number and expression, there are 21 genes in the 8p11-p12 region that can be considered as candidate breast cancer oncogenes. Included in this list are genes previously identified by us and others, such as *LSM1, BAG4*, and *SPFH2*. The list also includes genes such as *POLB, VDAC3, LETM3*, and *EIF4EBP1* that have not been previously implicated in breast cancer development.

**Transforming activity of candidate oncogenes and identification of oncogene interactions in the 8p11-p12 amplicon.** Given the number of plausible candidate genes identified based on their expression status when amplified, it is important to develop experimental approaches that allow for direct analysis of the transforming function of candidate oncogenes and can detect potential gene interactions within an amplicon. Therefore, we designed a cell transformation strategy that makes use of the growth factor dependency of the MCF-10A human mammary epithelial cell line (Fig. 3*A*). In previous work, we showed the ability of bona fide breast cancer oncogenes to transform MCF-10A cells to growth factor independence, and we have used transformation to growth factor independence to design an expression cloning strategy that can detect the transforming function of oncogenes from a defined library of genes (33, 35–38). To begin to analyze the transforming function of genes in the 8p11-p12 amplicon, we focused on the genes in the core region of gene amplification as defined in these and previous studies. We chose eight genes from this region based on their statistical association between copy number increase and expression level, or based on previous associations with transforming function when overexpressed, and prepared lentiviral expression constructs for each gene. Each lentiviral vector was packaged, titered, and combined before infecting midconfluent cultures of MCF-10A cells. Forty-eight hours after infection, MCF-10A cells were switched to media devoid of either insulin, EGF, or both insulin and EGF and cultured for 2 to 3 weeks. Colonies that emerged in growth factor–free media were propagated continuously in the same media, and those that exhibited continuous growth factor–independent proliferation were characterized further. To determine which genes from the library were expressed in growth factor–independent clones, RT-PCR analysis was done using primers specific for the library genes and for the vector.

Table 2 shows the results of experiments aimed at detecting the acquisition of insulin-independent growth capacity in library-infected cells. As shown in the table, three genes (*C8orf4, BAG4*, or *LSM1*) were consistently present in the insulin-independent clones isolated. Furthermore, one or more of these three genes were detected in every insulin-independent clone recovered. By contrast,

**Table 1.** Statistically significant association between copy number and expression in top 21 genes

| Gene | Spearman | Unadjusted *P* | Holm's adjusted *P* | Benjamini and Hochberg's adjusted *P* |
|---|---|---|---|---|
| *SPFH2* | 0.82 | 0.000001 | 0.00002 | 0.00002 |
| *PROSC* | 0.80 | 0.000001 | 0.0001 | 0.00003 |
| *DDHD2* | 0.81 | 0.000004 | 0.0002 | 0.00005 |
| *BAG4* | 0.79 | 0.000003 | 0.0001 | 0.0001 |
| *TM2D2* | 0.72 | 0.00004 | 0.002 | 0.0003 |
| *FUT10* | 0.73 | 0.00004 | 0.002 | 0.0003 |
| *BRF2* | 0.73 | 0.00004 | 0.002 | 0.0003 |
| *RAB11FIP1* | 0.73 | 0.00004 | 0.002 | 0.0003 |
| *LSM1* | 0.69 | 0.0001 | 0.005 | 0.001 |
| *AP3M2* | 0.67 | 0.0002 | 0.01 | 0.001 |
| *POLB* | 0.64 | 0.0005 | 0.02 | 0.002 |
| *PPAPDC1B* | 0.64 | 0.0005 | 0.02 | 0.002 |
| *FGFR1* | 0.64 | 0.0007 | 0.02 | 0.003 |
| *ASH2L* | 0.63 | 0.001 | 0.03 | 0.003 |
| *C8orf41* | 0.65 | 0.001 | 0.04 | 0.004 |
| *AGPAT6* | 0.59 | 0.002 | 0.06 | 0.01 |
| *LETM2* | 0.57 | 0.003 | 0.09 | 0.01 |
| *WHSC1L1* | 0.56 | 0.003 | 0.10 | 0.01 |
| *VDAC3* | 0.56 | 0.004 | 0.11 | 0.01 |
| *HOOK3* | 0.56 | 0.004 | 0.11 | 0.01 |
| *EIF4EBP1* | 0.54 | 0.005 | 0.14 | 0.01 |

the remaining five genes from the library (*ZNF703, RAB11FIP1, FGFR1, PPAPDC1B*, and *EIF4EBP1*) were only detected in 4 or 5 of the 20 clones examined, and each of those clones also expressed either *C8orf4, BAG4*, or *LSM1* (see Supplementary Table S2 for insert data on each clone). These results suggested that C8orf4, BAG4, and/or LSM1 were primarily responsible for the acquisition of insulin-independent growth of MCF-10A cells. To confirm these results, MCF-10A cells were infected with *C8orf4, BAG4*, or *LSM1* lentiviral vectors individually, and as shown in Table 2*B*, all three genes did, indeed, yield clones that could grow in the absence of insulin. Similarly, infection of MCF-10A cells with each of the other five constructs individually never gave rise to any insulin- or EGF-independent clones. Thus, three of the eight genes examined could induce insulin-independent growth, but none of the genes by themselves resulted in EGF-independent proliferation.

Next, MCF-10A cells were infected with the lentiviral library and selected for growth in EGF-free medium. In this experiment, many EGF-independent colonies emerged. Table 2 shows that the EGF-independent colonies that emerged contained predominantly the same three genes that were detected in the insulin-independent colonies. However, all EGF-independent colonies contained two or more of the genes previously shown to induce insulin-independent growth when expressed alone (see Supplementary Table S2 for inserts in individual clones). In addition, the remaining five genes in the library were rarely present in the EGF-independent clones and, when present, were always in clones that expressed at least two of the previously implicated genes. Taken with the previous results, which indicated that C8orf4, BAG4, or LSM1 can induce insulin-independent but not EGF-independent growth when expressed alone, these results strongly suggested that the same three genes can act in combination to transform cells to EGF independence. To confirm this observation, MCF-10A cells were infected with lentiviral vectors for *LSM1, BAG4*, and *C8orf4* in all combinations of two or three and selected directly for growth in EGF-free media. The results of this experiment are summarized in Table 2*B* and confirmed the results of the library experiments. In this study, any combination of LSM1, BAG4, and C8orf4 resulted in cells that could grow in EGF-free media. Interestingly, however, none of the gene combinations resulted in cell growth in the absence of both insulin and EGF.

**Characteristics of oncogene-transformed MCF-10A cells.** To characterize further the transformed phenotypes exhibited by MCF-10A cells expressing *LSM1, BAG4*, and/or *C8orf4*, we examined soft agar growth and determined the requirement for growth factor receptor activation in factor-independent proliferation. Figure 3*B* and *C* shows that, whereas parental MCF-10A cells have no capacity for growth in soft agar, both insulin-independent and EGF-independent transformants formed numerous colonies in agar. In this assay, the highest level of agar colony-forming efficiency was observed in the EGF-independent cells expressing all three oncogenes. These results confirm that cells selected based on growth factor independence exhibit other transformed phenotypes *in vitro*, and that oncogene interaction plays a role in expression of these phenotypes.

Next, growth factor–independent cells were characterized for the activation of signaling receptors that could mediate the growth factor independence phenotype. Figure 4*A* shows that all of the EGF-independent clones isolated expressed constitutively tyrosine phosphorylated EGFR. By contrast, control MCF-10A cells expressed tyrosine phosphorylated EGFR only in the presence of exogenous EGF. In addition, exposure of these EGF-independent cells to the EGFR tyrosine kinase inhibitor Iressa (gefitinib) resulted in complete growth inhibition, indicating the necessity of constitutive EGFR activation for the growth of these transformed cells (Fig. 4*B, left*). The presence of constitutively activated EGFR in the EGF-independent cells suggests the presence of an autocrine factor that is driving growth in the absence of exogenous EGF. To examine this question, conditioned medium was collected from the

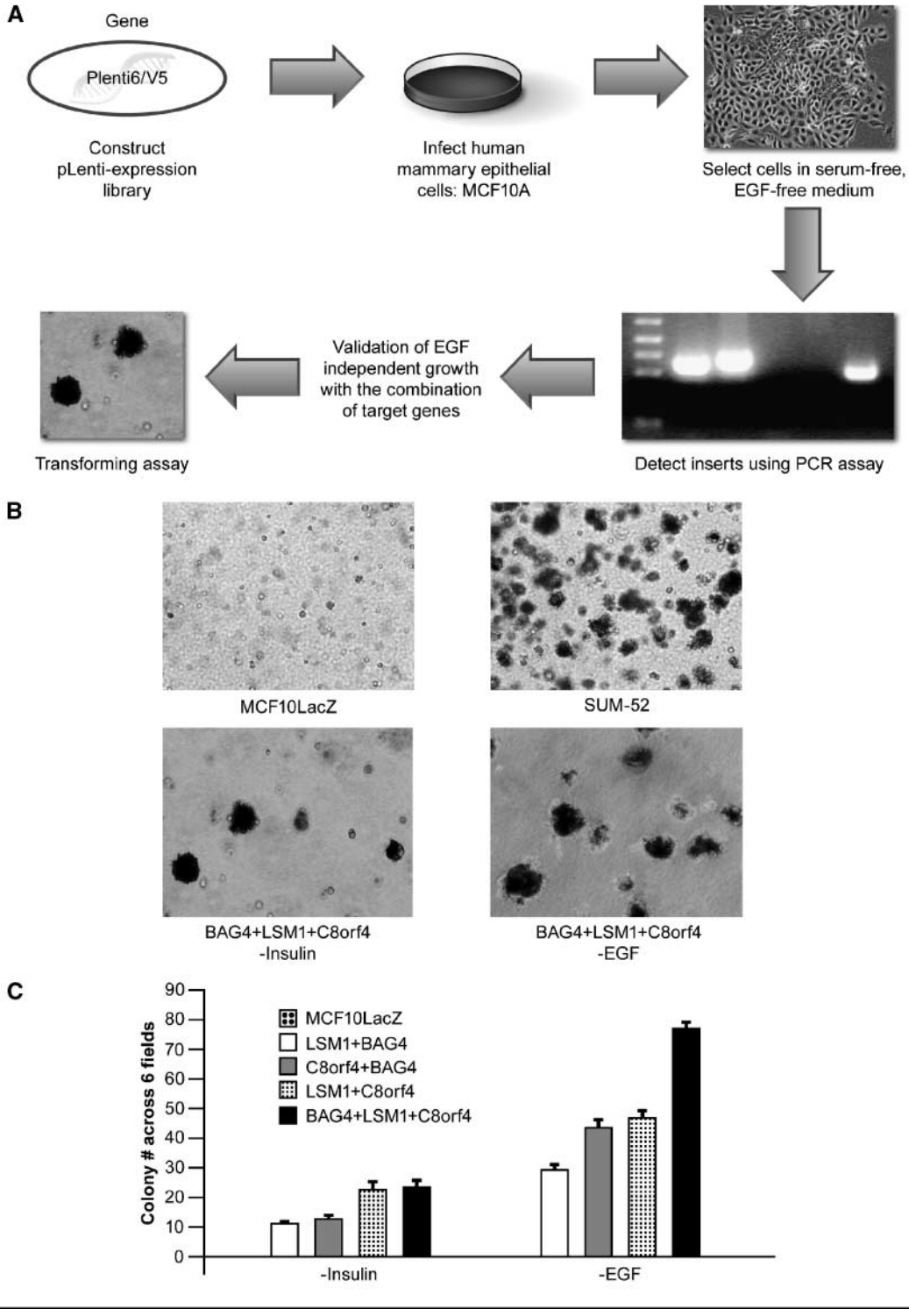



**Figure 3.** Infection with *LSM1, BAG4*, and *C8orf4* gene combinations induces EGF-independent growth and soft agar colony formation. *A,* schematic of the strategy to identify and validate oncogenes and oncogene combinations from the chromosome 8p11-p12 region that induce the growth factor independence and anchorage-independent growth of MCF-10A cells. Lentiviral expression constructs were created for candidate oncogenes from the 8p11-12 amplicon and sequenced to ensure their accuracy. Virus of the same titer from all candidate genes was combined and used to infect MCF-10A human mammary epithelial cells. Recipient cells were selected for the presence of insert with 10 μg/mL blasticidin and functionally selected for their ability to proliferate in growth factor–deficient medium. RNA was isolated from the resulting clones, and the presence of various inserts was evaluated by RT-PCR using vector and insert-specific primers. Factor-independent growth with the combination of target genes was validated by re-infecting MCF-10A cells and characterizing the resulting transformed phenotypes. *B,* MCF-10A cells overexpressing combinations of LSM1, BAG4, and C8orf4 grown in insulin-independent (*−insulin*) or EGF-independent (*−EGF*) conditions were added to a 0.3% agarose solution and plated into six-well plates containing a layer of 1% agarose. After 3 weeks, excess medium was removed, and 500 μg/mL solution of *p*-iodonitrotetrazolium violet was added to the wells overnight to stain for viable cells. *C,* viable colonies were quantified in MCF-10A cells infected with all combinations of LSM1, BAG4, and C8orf4 by counting colonies in six fields per sample in duplicate. Experiments were repeated twice. *Columns,* means; *bars,* SE.

**Table 2.**

A. *BAG4, LSM1*, and *C8orf4* are preferentially present in insulin-independent and EGF-independent clones following a multiple gene infection strategy

| Gene | Insulin-independent clones with insert | EGF-independent clones with insert |
|---|---|---|
| *BAG4* | 14/20 | 8/10 |
| *LSM1* | 13/20 | 10/10 |
| *C8orf4* | 13/20 | 9/10 |
| *EIF4EBP1* | 4/20 | 2/10 |
| *PPAPDC1B* | 5/20 | 2/10 |
| *FGFR1* | 2/20 | 1/10 |
| *ZNF703* | 5/20 | 3/10 |
| *RAB11FIP1* | 4/20 | 4/10 |

B. BAG4, LSM1, and C8orf4 cooperate to induce EGF-independent growth

| Selection conditions | Gene insert | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Empty vector | *LSM1* | *C8orf4* | *BAG4* | *LSM1 + C8orf4* | *LSM1 + BAG4* | *C8orf4 + BAG4* | *LSM1 + C8orf4 + BAG4* |
| +BL | + | + | + | + | + | + | + | + |
| +BL, −Insulin | − | + | + | + | + | + | + | + |
| +BL, −EGF | − | − | − | − | + | + | + | + |
| +BL, −Insulin, −EGF | − | − | − | − | − | − | − | − |

NOTE: Lentiviral expression constructs were created for candidate oncogenes from the 8p11-p12 amplicon, including *LSM1, EIF4EBP1, C8orf4, PPAPDC1B, ZNF703, RAB11FIP1, BAG4*, and *FGFR1*. 293FT cells were transfected with each lentiviral vector, and virus was harvested 48 hours after transfection. Virus of the same titer from all eight genes was combined and used to infect MCF-10A human mammary epithelial cells. To select for the presence of insert(s), 10 μg/mL blasticidin was added to MCF-10A cells 48 hours after infection, as well as media without insulin or without EGF to determine gene combinations that lead to growth factor independence. A, RNA was isolated from 20 clones growing in insulin-independent medium resulting from four separate infections or from 10 clones growing in EGF-independent medium resulting from three separate infections. The presence of various inserts was evaluated by RT-PCR using insert-specific primers. B, MCF-10A cells were infected with *C8orf4, BAG4*, or *LSM1* lentivirus individually, two at a time, or in a combination of three. Selection medium with 10 μg/mL blasticidin and without insulin, EGF, or both was added 48 hours after infection. −, no colony growth; +, colony growth under the given conditions.
Abbreviations: BL, blasticidin; −EGF, EGF independent; −Insulin, insulin independent.

EGF-independent MCF-10A variants and tested for its ability to substitute for exogenous EGF in parental MCF-10A cells. The data in Fig. 4*B* (*right*) show that conditioned medium from EGF-independent cells has substantial EGF-like growth factor activity that is likely responsible for EGFR activation and growth in EGF-free media. Furthermore, the addition of Iressa to conditioned medium from EGF-independent cells prevented MCF-10A growth, suggesting that its EGF-like growth factor activity depends on EGFR activation (Fig. 4*B, right*).

In contrast to the results obtained with EGF-independent cells, MCF-10A transformants growing in insulin-free media showed no signs of constitutive activation of IRS-1, the main signaling molecule activated by the IGF-I receptor (Fig. 4*C*). However, low-level insulin-replacing activity was detected in conditioned media derived from these cells, suggesting the presence of a non-IGF family growth factor present in conditioned medium that may play a role in the insulin-independent growth of the MCF-10A transformants (Fig. 4*D*).

**Oncogene expression related to clinical variables of the breast cancer panel.** The data (Supplementary Table S3) show the demographic and clinical variables of the 90 breast cancers in our original panel separated by the presence or absence of copy number increases at 8p11-p12. Consistent with results from other laboratories, there were no clear clinical features that were associated with gene amplification in this region. Because we do not have outcome data associated with our panel of breast cancer specimens, we were not able to analyze the role of amplification to variables of disease progression. However, in the recent work of Gelsi-Boyer et al. (31), there was a statistically significant association of decreased metastasis-free survival and the presence of the 8p11-p12 amplicon.

In summary, our results suggest that there are as many as 21 genes in the 53 gene region that are overexpressed in breast cancer when their copy numbers are increased. The large number of candidate oncogenes identified using statistical approaches illustrates the importance of using functional assays to identify the true transforming oncogenes. In that regard, our results provide the first evidence for cooperation among the LSM1, BAG4, and C8orf4 oncogenes that alters the transformed phenotypes of mammary epithelial cells.

## Discussion

The 8p11-p12 chromosomal region has been the subject of significant interest, particularly in human breast cancer where

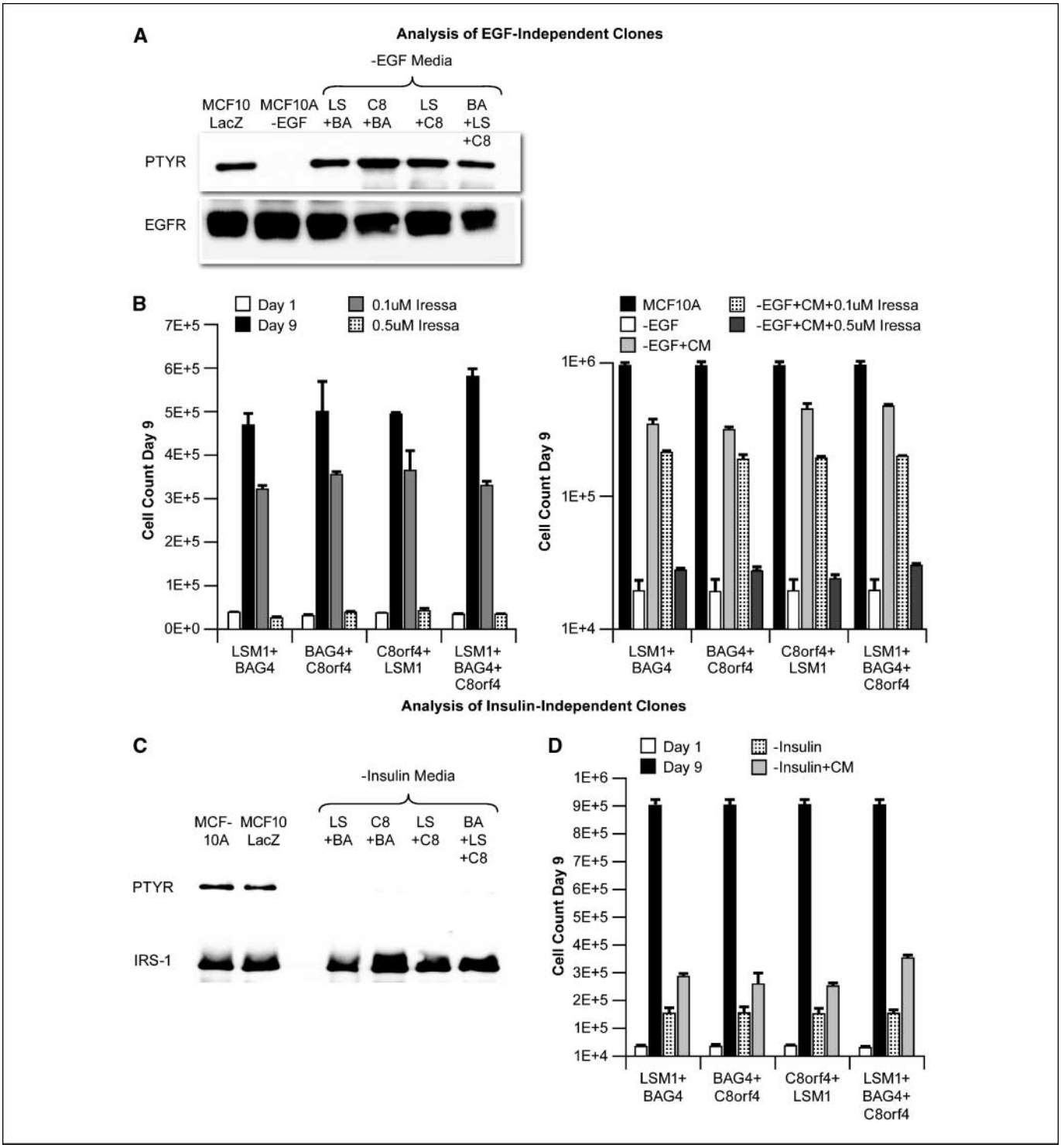



**Figure 4.** Involvement of IGF-1R and EGFR pathways in insulin-independent and EGF-independent clones resulting from overexpression of combinations of LSM1, BAG4, and C8orf4. *A,* whole-cell lysates were collected from MCF-10A cells infected with control vector or with combinations of *LSM1, BAG4*, and *C8orf4* genes. To determine EGFR phosphorylation levels, an EGFR immunoprecipitation followed by Western blots for phosphotyrosine or EGFR were done. Removal of EGF for 24 hours was used as a negative control for EGFR phosphorylation. Experiments were repeated at least twice. Representative blot. *LS,* LSM1; *BA,* BAG4; *C8,* C8orf4. *B, left,* cells were seeded in six-well plates and grown in SFIH medium. Cells were either vehicle treated or treated with 0.1 or 0.5 μmol/L of the EGFR inhibitor Iressa every 24 hours. After 9 days, cell counts were determined using a Coulter counter. Experiments were repeated at least twice. *Columns,* means; *bars,* SE. *Right,* conditioned medium from subconfluent MCF-10A cells infected with gene combinations growing in EGF-independent medium was collected 48 hours after feedings. MCF-10A cells were seeded in six-well plates and grown in SFIH medium, SFIH supplemented 1:1 with conditioned medium, or SFIH supplemented 1:1 with conditioned medium + 0.1 or 0.5 μmol/L Iressa added every 24 hours. After 9 days, cell counts were determined using a Coulter counter. Experiments were repeated at least twice. *Columns,* means; *bars,* SE. *C,* to determine IRS-1 phosphorylation levels, an IRS-1 immunoprecipitation followed by Western blots for phosphotyrosine or IRS-1 were done. Experiments were repeated at least twice. Representative blot. *D,* MCF-10A cells were seeded in six-well plates and grown in SFHE medium or SFHE supplemented 1:1 with conditioned medium collected from subconfluent MCF-10A cells infected with oncogene combinations growing in insulin-independent medium. After 9 days, cell counts were determined using a Coulter counter. Experiments were repeated at least twice. *Columns,* means; *bars,* SE.

focal copy number increases occur in ~15% of cases (27, 31, 39). Thus, it is highly likely that one or more breast cancer oncogenes reside in this genomic region. In our original experiments using three breast cancer cell lines developed in our laboratory, we provided evidence that the previously suspected candidate oncogene (*FGFR1*) was not the only oncogene candidate and, indeed, may be among the least interesting genes from this region, at least in breast cancer. In addition, we provided evidence for a role of *LSM1, ZNF703, RAB11FIP1, PPAPDC1B, C8orf4*, and *TACC1* in breast cancer development (32).

Based on our previous findings, we set out to validate and extend these observations using a panel of 100 primary human breast cancer specimens. The results obtained in these experiments now point to several genes that are very likely to play important roles as bona fide breast cancer oncogenes. In addition, we now provide some of the first functional evidence for a transforming role for these genes, when overexpressed either alone, or in specific combinations.

Based on our statistical analysis of the correlation between copy number increase and expression levels of each of the 53 genes in the 8p11-p12 region compared with three different sources of human mammary cell RNA, we identified 21 genes that were overexpressed in association with copy number increase at the $P < 0.01$ level (see Table 1). Several of these genes had been implicated as oncogenes in our previous experiments, including *LSM1, RAB11FIP1, PPAPDC1B*, and *EIF4EBP1*. By contrast, other genes that we suspected could play a role as oncogenes in the cell lines did not make our list of candidate genes in the present studies, including *TACC1* and *C8orf4*. Recent data from other laboratories also are not consistent with an oncogenic role for TACC1 in breast cancer and suggest it to be a candidate tumor suppressor gene from this genomic region (40). *C8orf4*, by contrast, seems to be overexpressed in a high proportion of breast cancers as well as thyroid and gastric cancer, regardless of copy number status (41, 42).

Recently, two groups reported results of copy number and gene expression analysis of the 8p11-p12 region in breast cancer. Gelsi-Boyer et al. (31) reported an extensive molecular cytogenetic analysis of 37 breast cancer cell lines and 62 primary breast cancers. They also analyzed the relationship between copy number and gene expression in 17 breast cancers. Garcia et al. (30) did a more focused analysis of 33 primary breast cancers and concentrated their studies on a 1-Mb core region of the amplicon common to most of the specimens in their panel. Thus, there are now three recent studies on the relationship between gene amplification and gene expression in over 50 primary breast cancers that have genomic alterations in this region. Several genes have been identified by all three groups as probable oncogenes based on statistical association between copy number and gene expression. These genes include *SPFH2, BRF2, RAB11FIP1, LSM1*, and *PPAPDC1B*. It is worth noting that Prentis et al. in their studies on the chromosomal rearrangements that occur near the *NRG1* locus also found that *SPHF2* was amplified and overexpressed in breast cancers with the 8p11-p12 amplicon (43). Several genes are common between our list of candidate genes and those identified by Gelsi-Boyer et al., including *PROSC, DDHD2, WHSC1L1, FGFR1, TM2D2(BLP1)*, and *AP3M2*. Finally, two genes were commonly identified by us and by Garcia et al, including *ASH2L* and *BAG4*. In addition to the genes commonly identified by our group and others, eight genes reached statistical significance in our study that were not mentioned in the studies of Garcia et al. or Gelsi-Boyer et al., including *FUT10, C8orf41(FLJ23263), EIF4EBP1, LETM2, AGPAT6, POLB, VDAC3*, and *HOOK3* (Supplementary Table S4).

The above discussion shows that statistical analysis of copy number and expression changes for individual genes within an amplicon can point to several genes that meet the criteria as candidate oncogenes. However, the number of genes identified and the variability in candidate oncogene lists obtained from different laboratories point to a clear need to validate candidate oncogenes based on transforming function. Accordingly, we tested the transforming activity of eight candidate oncogenes from the 8p11-p12 region using well-established methods in our laboratory.

The genes we chose to examine (*LSM1, BAG4, RAB11FIP1, PPAPDC1B, ZNF703, EIF4EBP1, FGFR1*, and *C8orf4*) were chosen based on several criteria. Most were chosen based on their statistical association between copy number and expression in our study, or on historical significance to the amplicon (e.g., *FGFR1*). *C8orf4* was included because of prior evidence from our laboratory, which indicated that C8orf4 overexpression plays an important role in the transformed phenotype of the SUM-52 cell line. The results obtained from these functional studies confirmed and extended our results with C8orf4 and showed its transforming potential towards MCF-10A cells. In addition, we found that both LSM1 and BAG4 can act individually to induce an IGF-independent phenotype and anchorage-independent growth capacity. In addition, by using an expression cloning strategy in which MCF-10A cells were infected with the entire mini-library of expression vectors, we were able to identify combinations of genes that induced an altered growth phenotype that was not induced by any of the genes when overexpressed individually. In previous studies from our laboratory, we have shown that the acquisition of EGF-independent growth potential is an indicator of highly transformed cells. In addition, the breast cancer cell lines that harbor the 8p11-p12 amplicon were originally isolated based on the ability to proliferate in growth factor–deficient media. Thus, growth factor independence is a hallmark of these breast cancer cell lines. Consistent with those previous findings, we showed that the three genes that were found to induce an insulin-independent growth phenotype when overexpressed individually could combine to render cells EGF independent. Thus, our first functional studies of candidate breast cancer oncogenes from the 8p11-p12 region provide strong evidence that *LSM1, BAG4*, and *C8orf4* are breast cancer oncogenes that have transforming function when overexpressed in human mammary epithelial cells.

*LSM1* has been previously implicated as a transforming oncogene in pancreas cancer (44), and more recent work has suggested that alterations in mRNA stability that occur when *LSM1* is overexpressed play a mechanistic role in its transforming function. *BAG4* has not been previously implicated as a transforming oncogene; however, this protein has been implicated in the radiation resistance of certain cancer cell lines, and its overexpression can prevent anoikis induced by blocking integrin signaling in normal epithelial cells (45–48). *C8orf4* is an interesting oncogene shown to be overexpressed in >90% of thyroid cancers (41, 42) and suggested to interact with Chibby, a negative regulator of WNT/β-catenin signaling (49, 50).

Perhaps as interesting as the genes that exhibited transforming function when overexpressed *in vitro* were some of the genes that were negative in this assay. First, FGFR1 overexpression did not result in expression of altered growth phenotypes, consistent with the results reported in our previous study. In contrast, previous work from other laboratories has shown a role for FGFR1 in prostate cancer progression (51, 52); in the regulation of transformation, proliferation, and migration of mouse mammary epithelial

cells (53, 54); and in myeloproliferative disorders caused by *FGFR1* gene translocations (55). Although our results are not consistent with an oncogenic role for *FGFR1* in breast cancers with an amplified 8p11-p12 region or in our model of growth factor independence, it is possible that FGFR1 may affect cancer progression in a way not specifically illustrated by our experiments and therefore merits continued investigation. In addition, *RAB11FIP1* and *PPAPDC1B*, which have been consistently implicated as candidate oncogenes based on their statistical association between amplification and expression and their biological function, had no transforming activity in our biological assays. Interestingly, PPAPDC1B has been recently implicated as a possible metastasis suppressor in hepatocellular carcinoma by showing that *PPAPDC1B* overexpression decreased invasion and metastasis with no effect on growth of the primary tumor (56).

In summary, the results reported here extend the previous studies by our laboratory and others, which implicate a number of key genes as transforming breast cancer oncogenes from the 8p11-p12 region. Our data not only implicate *LSM1, BAG4*, and *C8orf4* as bona fide oncogenes but have shown the potential for oncogene interactions within an amplicon in human breast cancer cells. Future studies will examine all 21 candidate oncogenes for transforming function, both singly and in combination, to examine how these genes interact with overexpressed genes from other amplicons within the same tumor specimen. This approach will ultimately result in the development of oncogene signatures that are likely to have important predictive power both for the natural history of disease progression and for predicting the best targeted therapeutic strategies.

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# Genomic organization of the 8p11~p12 amplicon in three breast cancer cell lines

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**Abstract** Amplification of chromosomal regions leads to an increase of DNA copy number and expression of oncogenes in human breast cancer (HBC). Amplification of the 8p11~p12 region occurs in 10–15% of primary, uncultured HBCs. In our panel of 11 breast cancer cells, three cell lines, SUM-44, SUM-52, and SUM-225, have overlapping amplicons in the 8p11~p12 region. To characterize genome structure of the amplified regions, we performed fluorescence in situ hybridization using 8p11~p12 BAC clones in the 3 cell lines. The results revealed that the 8p11~p12 amplicon has a highly complex structure and that *FGFR1* is not in the common core-amplified domain in 3 breast cancer cell lines with the amplicon. These 3 cell lines provide good models for genetic and functional studies of candidate oncogenes of the 8p11~p12 region. 

## 1. Introduction

Genomic amplification is often observed in many types of human tumors, including human breast cancer (HBC). Oncogenes such as *ERBB2* (17q12), *CCND1* (11q13), and *C-MYC* (8q24), are activated by amplification and play a role in the development of some fraction of HBCs [1–4]. Recently detailed analyses of genomic structures and sequences of amplified regions such as those found at 11q13, 17q12~q23, and 20q12 have revealed that amplicons have complex patterns and frequently involve non-syntenic as well as syntenic DNA from the same chromosomal region and can harbor multiple genes likely to be associated with tumorigenesis [5–7]. In breast cancer, the 11q13 amplicon can vary in size from less than 1 to 4.5 Mb. This amplicon includes a number of candidate oncogenes including *CCND1* and *EMS1*, which can be amplified independently of each other [2,5]. Similarly, there appears to be two distinct regions on 17q that become amplified in breast cancer; the 17q11 region that harbors *HER-2* and other candidate oncogenes, and the 17q23 region that has been recently described by different laboratories [6,8].

Tumor cell lines are good models for fine mapping of amplified genomic regions and for functional studies of candidate oncogenes because their molecular and cytogenetic aberrations and biological properties reflect distinct subsets of primary tumors. Over the past several years, we have developed a novel panel of HBC cell lines that are ideally suited for elucidating molecular biologic characteristics of breast cancer [9]. In our panel of 11 breast cancer cell lines, we found 3 cell lines, SUM-44, SUM-52, and SUM-225, that have overlapping amplicons centered at 8p11.2. Amplification of the 8p11~p12 region occurs in 10–15% of primary, uncultured HBCs [10], and fibroblast growth factor receptor 1 (*FGFR1*) has long been considered to be the best candidate oncogene at that locus. However, the exact involvement of this receptor in the progression of the cancer is unclear because it is not consistently present in the core-amplified domain and is not always overexpressed when amplified. Identification and characterization of amplified regions can provide important insights into the pathogenesis of breast cancer, and can lead to the identification of targets for novel therapeutics. In this report, we describe in detail the genomic structure of the 8p11~p12 amplicon with molecular cytogenetic analysis. Like other amplicons identified in breast cancers, the 8p11~p12 amplicon has a highly complex

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structure, and *FGFR1* is not in the common core-amplified domain in 3 breast cancer cell lines with the amplicon.

## 2. Materials and methods

Our previous chromosome and array comparative genomic hybridization (CGH) studies demonstrated that three of our breast cancer cell lines, SUM-44, SUM-52, and SUM-225 exhibited high-level amplifications at 8p11~p12 [9,11] Furthermore, the SUM-225 cell line contains a separate amplicon at 8q11. The array CGH results and the detailed map of the 8p11~p12 and 8q11 amplicon in SUM-44, 52, and 225 based on the April 2003 freeze of the human genome sequence (UCSC) is shown in Table 1.

In an effort to more carefully define the genomic structure of the amplicons in these three cell lines, and to better understand the mechanistic basis for the copy number increases observed in them, fluorescence in situ hybridization (FISH) analyses were carried out using BAC probes that map to the 8p11~p12 and 8q11 regions. We selected five commercially available BACs from the completed chromosome 8 sequence as probes for the 8p11~p12 region: RP11-701H6 (*ADRB3* locus), RP11-350N15 (*FGFR1* locus), RP11-723D22 (*TACC1* locus), RP11-44K6 (*INDO* locus), and RP11-470M17 (*TC-1* locus) (Fig. 1). One BAC, RP11-217N16, from the 8q11.1 region was also used in the SUM-225 line. A chromosome 8 centromeric probe, CEP8, was used as a control.

Metaphase chromosome slides were prepared from the SUM series of HBC cell lines using standard methods. Chromosomal in situ suppression hybridization and fluorescent detection of hybridization signals were carried out as described previously [12]. The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes.

## 3. Results

In the SUM-44 cell line, all 8p11~p12 BACs yielded clustered FISH signals on 2 marker chromosomes (Fig. 2). In addition, 2 apparently normal copies of chromosome 8 were present in most metaphase spreads. By contrast, only 4 FISH signals on most metaphase and interphase spreads were detected with the centromeric (CEP8) probe. In metaphase spreads, the hybridization pattern of the BAC probes and CEP8 signals were always located on the same marker chromosomes with 8p11~p12 amplification. FISH combined with array CGH analysis revealed the amplified 8p11~p12 region in SUM-44 to be intrachromosomal and to involve several megabases of syntenic sequences (Figs. 1–2). Based on the FISH hybridization pattern and our previously published karyotype of SUM-44 [13], we suggest a simple primary structural model of the 8p11~p12 amplicon in this cell line. During the tumorigenic process, the 8 p11~p12 region of one chromosome 8 underwent in situ amplification resulting

Table 1
Mapping profile of array-CGH within 8p11~12 and 8q11 region in 3 cell lines

| BAC Clone | Cytoband | Gene | Base start | Base end | SUM-44 | SUM-52 | SUM-225 |
|---|---|---|---|---|---|---|---|
| RP11-139G9 | 8p12 | *WRN* | 30886709 | 30928744 | −0.72 | −0.84 | −1.30 |
| CTD-2020E14 | 8p12 | | 31062838 | 31063263 | −0.73 | −0.82 | −1.42 |
| RP11-5713 | 8p12 | *NRG1* | 32268599 | 32429032 | −0.77 | **1.81** | −1.45 |
| RP11-122D17 | 8p12 | *NRG1* | 32435232 | 32609814 | −0.83 | **1.88** | −1.51 |
| RP11-258M15 | 8p12 | | 33377743 | 33538816 | −0.73 | **2.34** | −1.26 |
| RP11-274F14 | 8p12 | *UNC5D* | 35328931 | 35329267 | −0.85 | **1.65** | −0.49 |
| RP11-237M13 | 8p12 | *UNC5D* | 35433788 | 35608399 | −0.77 | **1.11** | −0.61 |
| RP11-210F15 | 8p12 | | 36250826 | 36251210 | ND | **1.02** | −0.64 |
| RP11-265K5 | 8p12 | *HTPAP*, *WHSC1L1*, *FLJ25409*, *FGFR* | 37849071 | 38026421 | ND | **1.28** | 0.69 |
| RP11-100B16 | 8p12 | *FLJ25409*, *FGFR1* | 37980628 | 38144207 | ND | **1.34** | 0.74 |
| RP11-262I23 | 8p11.21 | *INDO* | 39468496 | 39667003 | **1.52** | **1.43** | **2.22** |
| RP11-133O7 | 8p11.21 | | 39578684 | 39626434 | **1.89** | **1.58** | **2.32** |
| RP11-284J3 | 8p11.21 | | 40384739 | 40385032 | **1.06** | **1.61** | −1.42 |
| RP11-282J24 | 8p11.21 | | 41386548 | 41386797 | **1.52** | **1.40** | −1.32 |
| RP11-64C22 | 8p11.21 | | 42090855 | 42125967 | 0.08 | **1.47** | −1.15 |
| RP11-73M19 | 8p11.21 | *FLJ32731*, *FLJ22242* | 42724620 | 42892080 | 0.07 | 0.72 | −1.22 |
| RP11-12L15 | 8q11.1 | | 47495880 | 47647566 | −0.03 | −0.12 | **3.36** |
| RP11-217N16 | 8q11.21 | | 47715320 | 47715660 | 0.08 | −0.08 | **3.34** |
| RP11-268N2 | 8q11.21 | | 48790973 | 48808994 | −0.57 | −0.03 | −1.07 |
| RP11-149G12 | 8q11.21 | | 51871742 | 51872129 | −0.39 | 0.12 | −0.78 |

The BAC clones in array-CGH are ordered by cytoband and base-pair position, and the log2 ration of each BAC probe is given for each cell line. Boldface indicates ratios greater than or equal to 1.00 as genomic amplification. The BAC's position and genes within each BAC clone were obtained from the UCSC database (http://genome.ucsc.edu).

*Abbreviation:* ND, not determined.

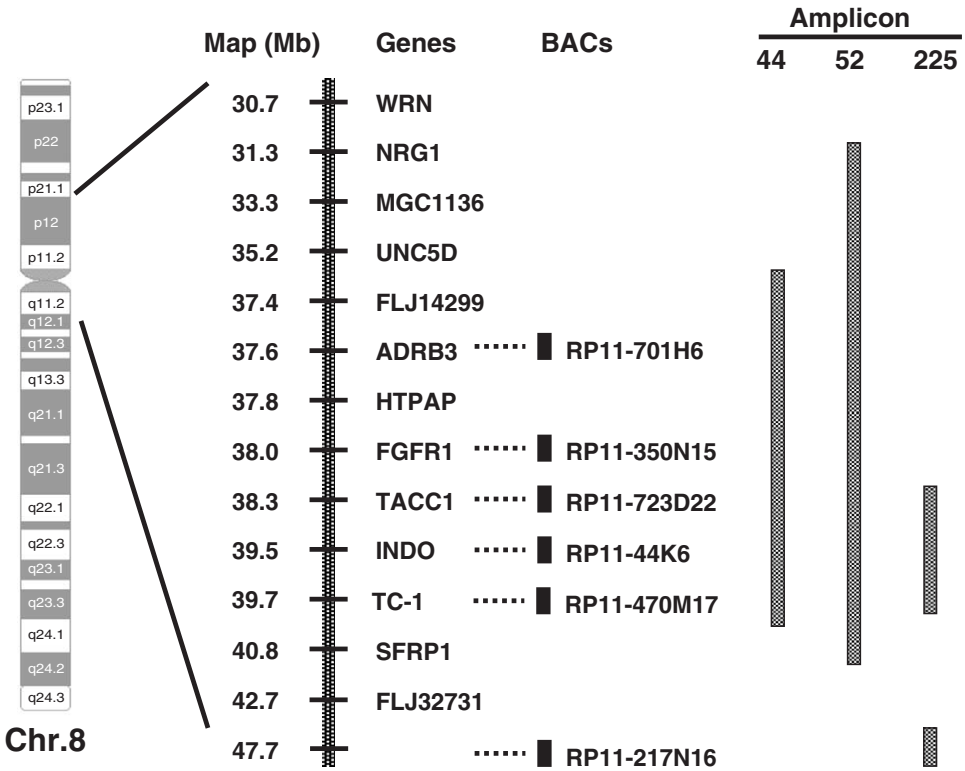


Fig. 1. Ideogram of 8p11~p12 amplicon in 3 HBC cell lines: SUM-44, SUM-52, and SUM-225. The position of representative genes based on UCSC database (http://genome.ucsc.edu), and BACs used for FISH are presented to the right of the chromosome 8 ideogram. The amplicon in each cell line is based on the results of array-CGH, FISH, and Southern blot analysis.

in a large tandem duplication of the region. Subsequently the chromosome containing the 8p11~p12 amplified chromosome and the normal chromosome 8 were duplicated resulting in two normal chromosome 8s and two chromosome 8s with the amplicon.

FISH patterns from SUM-225 cells resembled those from SUM-44 cells in that copy number increases appear to have resulted from the generation of clustered DNA amplification. However, the regions of focal gene amplification in SUM-225 differ from those of SUM-44 cells. In the SUM-225 cell line, FISH (Fig. 3) and array-CGH (Table 1) analysis revealed two separate amplified regions; one at 8p11~p12 and a second at 8q11.

In metaphase spreads, clustered FISH signals obtained with the 44K6 and 470M17 probes were present on 2–4 marker chromosomes (Fig. 3, top left panel) and more than 18 FISH signals were counted in interphase spreads, while BAC probe 723D22 (*TACC1* locus) yielded 7 to 12 signals in metaphase and interphase chromosome spreads. In contrast, probes 701H6 (Fig. 3, bottom right panel) and 350N15 yielded essentially background signals. Interestingly, BAC probe 217N16, located at 8q11.1, also showed high-level amplification in metaphase and interphase nuclei (Fig. 3, bottom left panel), and clustered FISH signals were also observed in 3–6 marker chromosomes in each metaphase spread.

Two-color FISH with 470M17 and 217N16 revealed the 8q11 and 8p11probes have coamplified FISH signals in 2–4 marker chromosomes, whereas the 8q11 probe has independent amplified signals in 1–3 other marker chromosomes. The BAC probe 217N16 from 8q11.1 actually overlaps with the centromere region, and therefore, it is not surprising that the centromeric CEP8 probe also showed increased signals on several marker chromosomes (Fig. 3, top right panel). This suggests that SUM-225 cells share a small common region of gene amplification with the SUM-44 and SUM-52 cells in the 8p11 region, and that they also have a separate unique region of gene amplification across the centromere involving the proximal 8q11 region. Because the 8q11 amplicon has the highest level of amplification in SUM-225 cell line, it may also harbor uncharacterized breast cancer genes. As we can only look at the end product of the amplification process, we do not know if the two amplicons within 8p11 and 8q11 regions of SUM-225 were initiated as a large amplified DNA fragment followed by a secondary rearrangement, resulting in two separate amplicons, or if the two domains were co-amplified in some marker chromosomes and independently in others.

The molecular cytogenetic alterations present in the SUM-52 cells are considerably more complex than for the SUM-44 or SUM-225 cell lines. Previous chromosome banding analysis showed SUM-52 had complex karyotypes

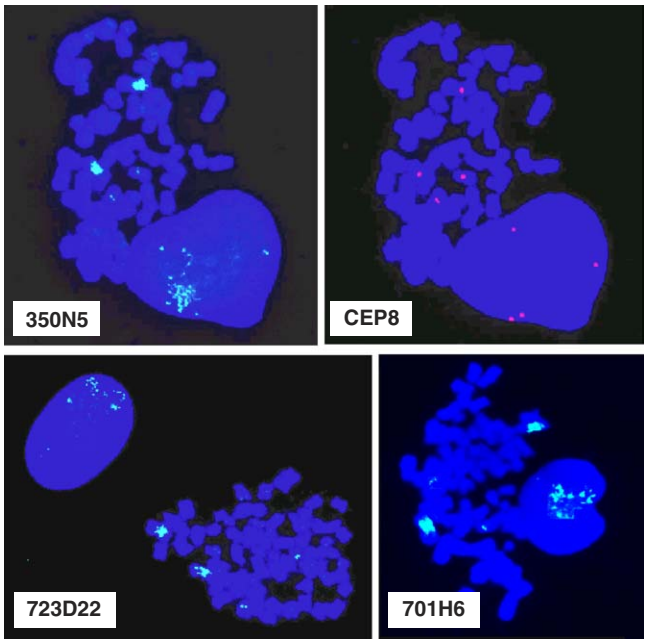


Fig. 2. Representative images of FISH analysis of the 8p11 amplicon demonstrate high-level amplification of the 8p11~p12 region in SUM-44 HBC cells. The BACs of 8p11~p12 region were amplified on 2 marker chromosomes and in more than 20 signals in interphase cells. There are only 4 chromosome 8 centromere signals (*red*) in the displayed metaphases and interphase spreads.

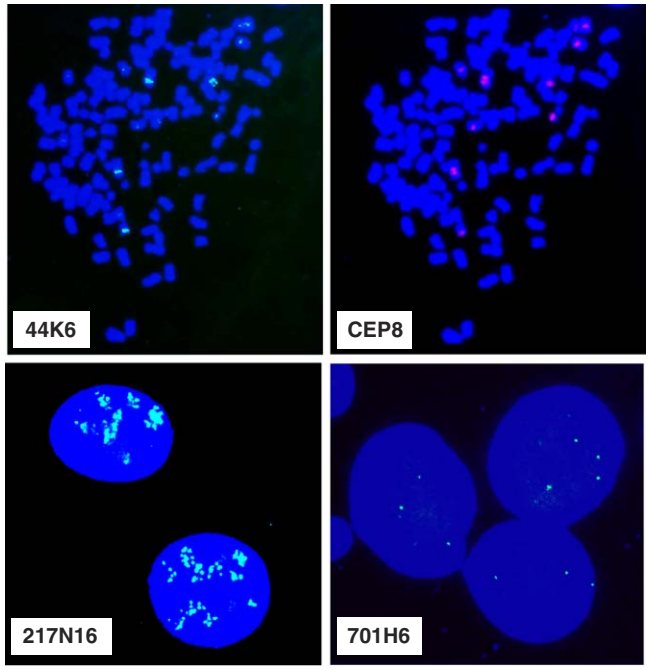


Fig. 3. Representative images of FISH analysis of the 8p11 amplicon demonstrate the two separate peaks of amplification in SUM-225 cells. The 44K6 BAC shows 8p11 amplification, however the 701H6 BAC does not show amplification. The centromeric probe and the 217N16 BAC from 8q11 also show amplification of sequences from the opposite side of the centromere at proximal 8q11.

with multiple numerical and structural aberrations [14]. The representative karyotype has one normal chromosome 8 with many marker chromosomes (http://www.cancer.med.umich.edu/breast_cell/Production/sumlines/karyotypes/Sum-52PE Karyotypes.html). In the FISH hybridization, the signal intensity and hybridization pattern of the five 8p11~p12 BAC probes in SUM-52 interphase and metaphase spreads were complex and heterogeneous. Twelve to 20 copies of the 5 BAC probes were detected in metaphase chromosomes and interphase nuclei, whereas 8 to 12 copies of the CEP8 probe were observed. In most metaphase spreads, intense signals of BAC probes were present on 1–3 marker chromosomes, suggesting clustered duplication of the corresponding genomic segment (Fig. 4c and d).

Interestingly, CEP8 signals were not detected on these marker chromosomes. However, BAC and CEP8 signals were detected in 2–3 cytogenetically normal chromosomes 8 (Fig. 4a). In addition, CEP8 signals were detected in 4–8 chromosomes that did not yield signals with 8p11~p12 BAC probes (Fig. 4b). These CEP8 signals were generally fainter and smaller than the corresponding normal centromeric signals. The fact that several derivative chromosomes showed only 8p11~p12 BAC or CEP8 signals in FISH hybridization, suggests that breaks and translocations in 8p11~p12 likely occurred during the amplification process. Indeed, Adelaide et al. has recently demonstrated that SUM-52 cells have 8p12 amplification and 8p12~pter loss with breakpoints in the NRG1 gene [15]. Thus, unlike SUM-44 and SUM-225 cells, SUM-52 cells have gene amplification as a result of more complex translocation and rearrangement. The presence of nonsyntenic amplified regions of the genome in the SUM-52 and SUM-225 cells suggests that the amplification process resulted from a combination of molecular events.

## 4. Discussion

Gene amplification is a frequent event in human cancers, but little is known regarding the mechanism of gene amplification or how the overall genomic structure that constitutes the amplified DNA is assembled. From the detailed studies of in vitro model systems of drug-resistant cell lines, it is generally agreed that at least two different mechanisms can drive amplification [16–18]. One is a breakage-fusion-bridge (BFB) cycle mechanism that accumulates copies organized as large repeats on a chromosome arm where one normal gene copy maps in non-amplified cells [16,19]. Second, the amplified DNA can megabase long extra-chromosomal DNA sequences called double minute. The BFB mechanism has proved relevant to the specific breakage of genomic DNA at fragile sites that are points at which chromosomes break non-randomly under certain specific conditions [18–21]. Coquelle et al. have found that fragile sites trigger intrachromosomal gene amplification and form the boundaries of amplicons [16]. In addition, gene amplification mediated by BFB cycles at fragile sites has been demonstrated

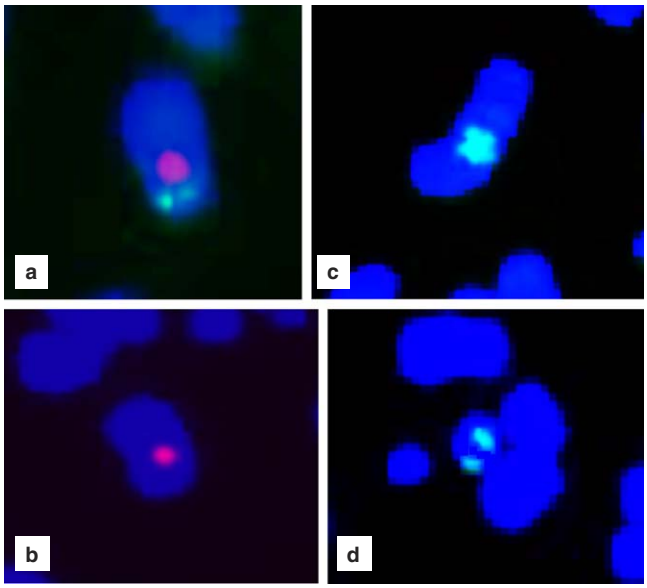


Fig. 4. Representative images of FISH analysis of the 8p11 amplicon demonstrate representative marker chromosomes from SUM-52 metaphase FISH experiments using the 350N15 BAC probe and the centromeric probe (CEP 8). Displayed are: an apparently normal chromosome 8 (*a*), a marker chromosome with only the CEP8 signal (*b*), and marker chromosomes with multiple signals from the 350N15 BAC probe (*c*,*d*).

in human cancer, such as for the *MET* oncogene in gastric cancer through FRA7G, and for the *RIN* gene in oral cancer through FRA11B [22,23].

The 8p11~q11 region has been reported as a common fragile site [24]. This suggests 8p11~p12 and 8q11 amplification in our cell lines appears to have resulted from BFB cycles at the 8p11~q11 fragile site. Our data from FISH, array-CGH, and Southern blot analyses, indicate that extensive DNA rearrangement and loss of intervening DNA may have taken place during the evolution of the 8p11~p12 amplification in SUM-52 and SUM-225 cell lines [11]. Our observations are in line with those made by Adelaide et al., who found the 8p12~p21 region is particularly complex with at least 7 different breakpoint targets within the *NRG1* gene in breast and pancreatic cancer cell lines [15]. The genesis of such complex abnormalities cannot be fully explained by BFB cycles and likely involves additional breakage and recombination events at both fragile sites and non-fragile site regions.

The results of the FISH studies were consistent with and extended the information gained from the conventional CGH and array-CGH analyses performed previously, and confirmed that *FGFR1* is beyond the core-amplified domain in SUM-225 cell line. Detailed expression profiling of the amplicon in our 3 breast cancer cell lines using a chromosome 8 cDNA microarray and northern blot analysis revealed that *FGFR1* is overexpressed at the message level only in the SUM-44 line [11]. Several genes including *TACC1*, *C8ORF4* (*TC-1*) and other genes within common core-amplified domain have been found to be overexpressed in breast cancer cell lines and primary tumors [11,25].

Our results suggest that the 8p11~p12 region, which has a similar complex amplification pattern as those observed at 20q12, 17q22~q24, and 11q13, may contain more than one important gene. Co-selection and a synergistic role of two or more genes may occur in the development and progression of some breast cancers. Further studies of the amplification and expression of candidate breast cancer oncogenes in a large set of primary breast cancers, as well as determination of their function in cell transformation, will be necessary to address the relationship between these genes and breast cancer progression.

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